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***Gene Expression Analysis of Telomerase Related  
Genes in Myeloid Malignancy***

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***Submitted in partial fulfilment of the requirements for the  
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## *Abstract*

Telomere shortening and an increased telomerase activity are associated with poor prognosis and disease progression in many cancers. In Chronic Myeloid Leukaemia (CML) telomere shortening has a strong correlation with disease progression. Expression of *hTERT*, the catalytic component of telomerase, was evaluated in the CD34<sup>+</sup> cells of CML patients. This revealed that expression of *hTERT* was significantly reduced in chronic phase CML and decreased with disease progression to accelerated phase and blast crisis. It could therefore be concluded that reduced *hTERT* expression contributes to reduced telomere length in CML. Additionally, expression of *c-Myc*, which increases *hTERT* transcription, correlated with *hTERT* expression suggesting decreased *hTERT* is partly caused by reduced *c-Myc*. *hTERT* promoter methylation and mutation status were investigated and this revealed that the *hTERT* promoter was not methylated and mutation rates were low suggesting that these are not contributing to reduced *hTERT* expression. cDNA microarrays were used to analyse gene expression in neutrophils of patients with Essential Thrombocythaemia (ET) harbouring the *JAK2* V617F mutation which, like the *BCR/ABL* translocation in CML, results in an activated kinase. Neutrophils of ET patients exhibited a gene expression profile close to that of controls despite the presence of the mutation. Affymetrix microarrays were used to investigate the role of telomerase related genes in Myelodysplastic Syndromes (MDS). Genes decreased in patients with del(5q) include positive regulators of telomere length and genes with higher expression were associated with increased telomerase activity. Inferring that in these patients telomere length would be reduced but counteracted by increased expression of genes promoting telomerase activity. Genes that could differentiate between RA and RAEB2 subtypes were mostly related to telomere

maintenance. These results demonstrate the complex role of telomerase and telomere length in malignancy and challenge the view that *hTERT* is always increased in cancer.

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# ***Chapter 1***

## ***Introduction***

### ***1.1 The Discovery of Telomeres and Telomerase***

The study of telomeres began in the 1930s with the proposals of Herman Müller and Barbara McClintock suggesting the presence of a structure to protect chromosome ends from fusing to one another (Muller 1938; McClintock 1941). Müller named these structures telomeres, from the Greek “telos” meaning end and “meros” meaning part.

The first telomere sequence to be discovered was that of *Tetrahymena thermophila* by Blackburn and Gall (Blackburn and Gall 1978). This was shown to be the tandemly repeated sequence CCCCTT. It was then demonstrated by Szostak and Blackburn that the telomeric sequences of *Tetrahymena* could protect DNA ends from fusion (Szostak and Blackburn 1982). By transfecting *Tetrahymena* telomere sequences into yeast on a linear plasmid they created a functional telomere that would replicate in yeast. These telomeres showed different lengths in a clonal population of yeast cells and did not fuse to form a circular plasmid, thus confirming the ideas put forward by Müller and McClintock. It was not until 1988 that the human telomere sequence was elucidated by Moyzis *et al.* (Moyzis *et al.* 1988). Fluorescent in situ hybridisation and nuclease digestion experiments allowed them to demonstrate that human telomeres consist of a tandemly repeated 5'-TTAGGG-3' sequence. These sequences were identified as functional human telomeres and serve to protect chromosome ends and solve the “end replication problem” (Watson 1972; Olovnikov 1973). This problem results from the semiconservative replication of DNA in the 5' to 3' direction and the use of short RNAs to prime DNA synthesis. This results in a loss of nucleotides at the 5' end of the DNA strand that cannot be filled and which would lead to the degradation of important genetic material if telomere DNA was not present. For human telomeres this gives rise to a shortening rate of about 50bp per chromosome end per cell division (Harley *et al.*

1990). If this erosion is not balanced by elongation then telomeres will shorten with each cell division. It was recognised that human cells are limited in the number of cell divisions they can undergo (Hayflick and Moorhead 1961). This limit is known as the Hayflick limit and is the phenomenon of replicative senescence. Senescence occurs when cells enter permanent growth arrest in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle while remaining metabolically active (Artandi and DePinho 2000). Telomere shortening leads to chromosome instability and the replicative senescence described by Hayflick.

Further work by Blackburn and Grieder on *Tetrahymena* revealed the presence of a ribonucleoprotein complex that was capable of de novo telomere synthesis (Greider and Blackburn 1987). They named this new reverse transcriptase Telomerase. Shortly after this discovery mutation of telomerase in yeast demonstrated that the enzyme was able to maintain the telomeric DNA at chromosome termini (Lundblad and Szostak 1989). Studies of the human cell line HeLa identified the presence of telomerase in human cells and its ability to add telomere repeats in these cells (Morin 1989). The enzyme was then implicated in human cellular senescence when work on human fibroblasts revealed that a loss of telomeric DNA was associated with serial passaging of the cells (Harley *et al.* 1990). Evidence that shortening of telomeres was the cause of cellular senescence was presented in 1998 by Bodnar *et al.* (Bodnar *et al.* 1998). They transfected the gene encoding the human telomerase reverse transcriptase enzyme (*hTERT*) into telomerase negative human cell lines and observed the restoration of telomerase activity leading to elongated telomeres and increased cell division when compared to the telomerase negative control cells which showed telomere shortening and senescence.

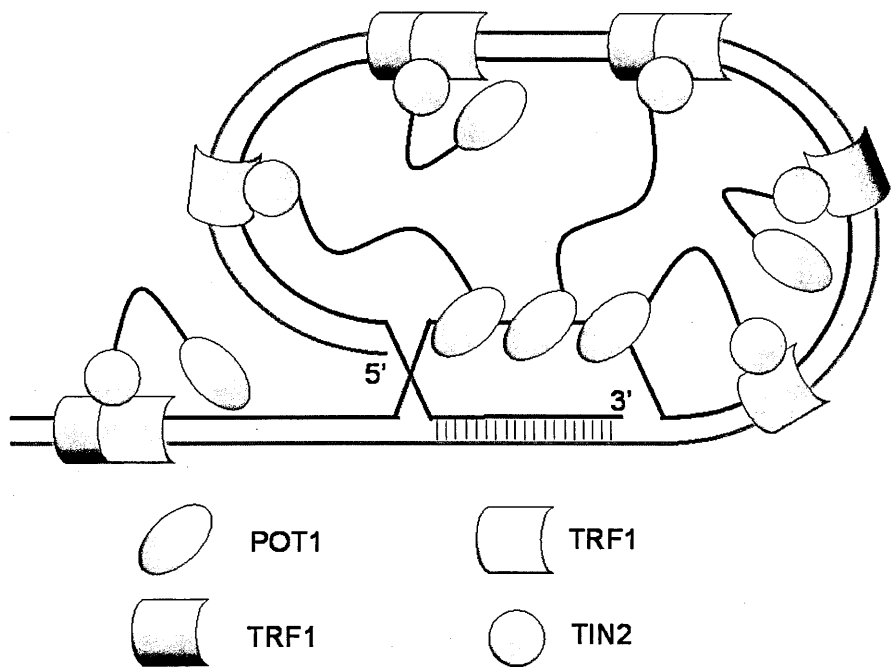
The importance of the work of Elizabeth Blackburn, Carol Greider and Jack Szostak was recognised in 2009 when they were awarded the Nobel Prize in physiology or medicine for their work on telomeres and telomerase.

### ***1.2 Structure of Human Telomeres***

Human telomeres consist of between 10 and 15 kb of double stranded telomeric TTAGGG repeats (Blasco 2005). At the terminal of the double stranded telomere there is a further single stranded 3' G-rich overhang of 100-200 nucleotides. This overhang has been shown, with the aid of electron microscopy, to loop back and anneal with the double stranded telomere region to form a "t-loop" structure (Griffith *et al.* 1999). It is thought that the t-loop forms when the single stranded 3' telomere overhang invades the double stranded region of the telomere repeats (Fig 1.1). This loop is proposed to protect the telomere ends by preventing the 3' overhang from being identified as a site of DNA damage or a double strand break (de Lange 2002).

Telomeres are bound by two telomere repeat binding factors known as TRF1 and TRF2 as represented in Fig 1.1. TRF1 binds double stranded telomere repeats and the longer the telomere the more TRF1 molecules that bind to the telomere (Smogorzewska *et al.* 2000). TRF1 is thought to function to limit telomere length by preventing telomerase from binding individual telomeres (van Steensel and de Lange 1997). This indicates that shorter telomeres would have reduced binding of TRF1 allowing telomerase to access the telomere repeats and lengthen shorter telomeres (Smogorzewska and de Lange 2004). In addition to its function of telomere length regulation TRF1 interacts with several telomerase related proteins. One of these is the enzyme Tankyrase, which is a poly(ADP-Ribose) polymerase (Smith *et al.* 1998). Tankyrase directly binds to

TRF1 at the telomere and ADP-ribosylates TRF1 to inhibit its binding to the telomere (Smith and de Lange 2000). This results in a reduction of TRF1 binding to telomeres and an increase in telomere elongation. Therefore, Tankyrase is a positive regulator of telomere length. The interaction between Tankyrase and TRF1 is modulated by TIN2. TIN2 stabilises the interaction between the two proteins and protects TRF1 from poly(ADP-Ribosylation) by tankyrase (Ye and de Lange 2004).



**Figure 1.1 Human telomere t-loop structure bound by telomere binding proteins.** Telomeric DNA is represented by the red and green lines and the single stranded 3' overhang is shown to be invading the double stranded region of telomere repeats. The displaced single stranded telomere repeats are bound by POT1 to maintain the t-loop structure (Yang *et al.* 2005). Figure modified from (Carroll and Ly 2009). Like TRF1, TRF2 also binds to double stranded telomere repeats, is more abundant on

long telomeres and negatively regulates telomere length (Smogorzewska *et al.* 2000). Overexpression of TRF2 leads to a reduction in telomere length suggesting a function similar to that of TRF1 of restricting elongation of telomeres (Smogorzewska *et al.* 2000). TRF2 has a second important function to protect the ends of individual telomeres and reduction of TRF2 results in loss of the G-strand overhang at telomere ends (van Steensel *et al.* 1998). TRF2 is thought to possess a DNA remodelling activity that would facilitate the formation of telomeres into the t-loop structure which can protect chromosome ends (Stansel *et al.* 2001; Khan *et al.* 2007). It is thought that loss of TRF2 results in reduced G-strand overhangs after DNA replication leading the chromosome ends to remain unprotected.

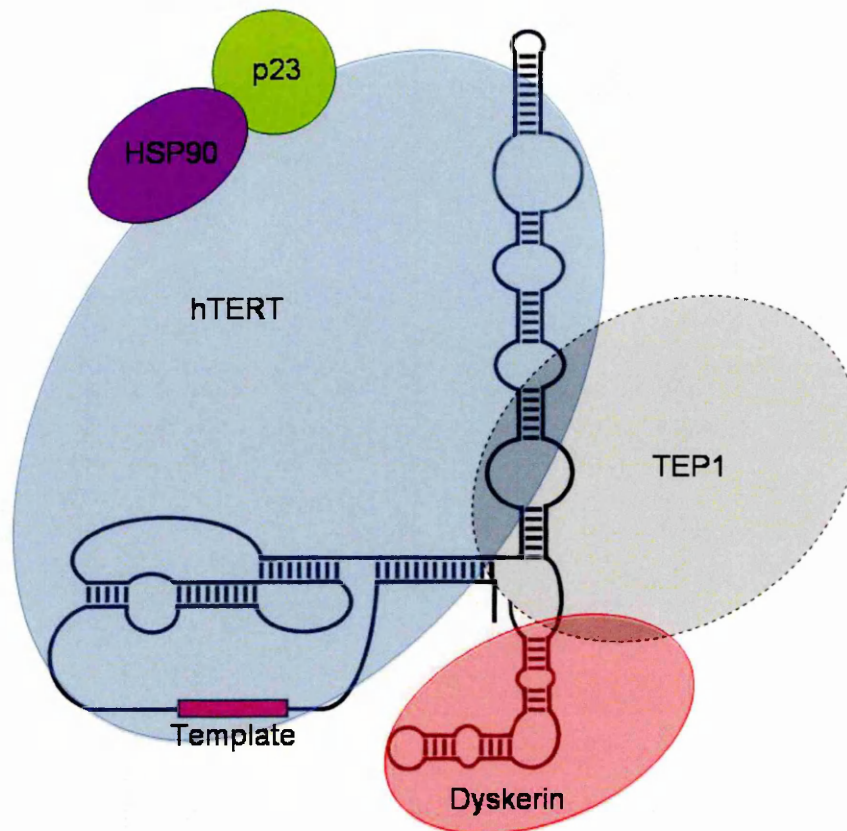
When telomeres become critically shortened the length of the G-strand overhang is reduced and this is thought to disrupt the formation of the T-loop (Stewart *et al.* 2003). In the absence of telomerase the telomeric overhang is not maintained and the telomeres become uncapped and unprotected by telomere binding proteins such as TRF2. These uncapped telomeres are recombinogenic and if a cell with critically shortened telomeres continues to divide unchecked then this may lead to chromosome end to end fusions as double strand break repair mechanisms are acting at telomeres (de Lange 2002). This can lead to chromosome breakages and other structural abnormalities such as loss or gain of parts of the chromosome which contribute to genomic instability (Boukamp *et al.* 2005). However, the cell possesses mechanisms to prevent these aberrations multiplying. Critically shortened telomeres with reduced overhangs and no TRF2 binding are recognised by the cell as dysfunctional and a DNA damage response is induced (Cosme-Blanco *et al.* 2007). This involves the well characterised tumour suppressor gene p53 is that is activated by DNA damage and cellular stress (Meek and Anderson 2009). Activation of p53 induces cellular senescence or cell death through



apoptosis both of which protect from tumour formation (Deng *et al.* 2008). The progressive telomere shortening observed in normal somatic cells leads to activation of p53 (Cosme-Blanco *et al.* 2007). This means that when the telomeres become sufficiently shortened further cell division is blocked, resulting in replicative senescence (Harley *et al.* 1990). This telomere shortening is thought to be a molecular mechanism to count the number of times a cell has divided (Wright and Shay 2001). Telomere sequences appear to have the function to protect human cells against the development of cancer by limiting the maximum number of permitted divisions (Sager 1991).

### ***1.3 Human Telomerase Reverse Transcriptase***

Human telomerase is a ribonucleoprotein complex that minimally consists of hTERT, the catalytic component, and hTR, the RNA template (Wenz *et al.* 2001). hTR is a short RNA template of 11 nucleotides that are complimentary to the telomere repeat sequence (Feng *et al.* 1995). hTERT is the reverse transcriptase subunit and the rate limiting factor for telomerase activity (Counter *et al.* 1998). Both the RNA template and the catalytic component are necessary for telomerase activity and without them both there is no functional enzyme activity (Wenz *et al.* 2001). Although sufficient for telomerase activity there is a requirement for accessory proteins in the telomerase holoenzyme complex to control enzyme assembly, regulation and activity.



**Figure 1.2 Human telomerase holoenzyme.** The secondary structure of the 451 nucleotide telomerase RNA component is represented as a solid black line with the 11 nucleotide telomere repeat template shown as a red box (Chen *et al.* 2000). hTERT, the catalytic component is represented in blue and shown interacting with the chaperone proteins Hsp90 and p23. Dyskerin is shown interacting with hTR to stabilize the RNA. The binding site of TEPI with hTERT and hTR is not known as present and it is thought that TEPI may have multiple binding sites on hTERT (Beattie *et al.* 2000).

Telomerase associated protein 1 (TEPI) is an RNA binding protein that interacts with the telomerase RNA component (Harrington *et al.* 1997). TEPI is not the rate limiting factor for telomerase activity but it is involved in enzyme activity and is thought to be required for full activity of the enzyme (Chang *et al.* 2002). TEPI also binds hTERT and is associated with telomerase activity although TEPI is not required to reconstitute telomerase activity (Beattie *et al.* 2000). As shown in Fig 1.2, TEPI assembles with hTERT, hTR, hsp90, p23 and dyskerin to form the telomerase holoenzyme complex

(Chang *et al.* 2002). Hsp90 and p23 are chaperone proteins that contribute to telomerase activity by aiding assembly of hTERT with hTR (Holt *et al.* 1999). Dyskerin binds hTR and has been implicated in stability of hTR (Mitchell *et al.* 1999). Interestingly, mutations of dyskerin lead to a form of the disease dyskeratosis congenita in which patients exhibit reduced telomerase activity, short telomeres, chromosome end to end fusions and increased incidence of cancer (Mitchell *et al.* 1999). During the telomerase reaction hTERT uses hTR as an RNA template to add single nucleotides to the 3' end of the telomere sequence resulting in telomere elongation (Blackburn *et al.* 1989). The access of telomerase to this 3' end is regulated by telomere repeat binding factors as described above.

#### ***1.4 Telomeres and Telomerase in normal human cells***

A sensitive method of detection of telomerase activity was developed by Kim *et al.* and is known as the TRAP assay (telomere repeat amplification protocol) (Kim *et al.* 1994). This has been developed into a PCR ELISA assay that detects the presence of telomerase in cells from the addition of telomeric repeats onto a synthetic primer. The PCR product then hybridises to a labelled probe which is detected by immunolabelling methods. Using this method it has been shown that telomerase is expressed during early human development and activity has been detected in most human somatic tissues up to 16-20 weeks of development (Wright *et al.* 1996). Telomerase activity is not detected in the majority of adult somatic tissues and in these cells where telomerase is not expressed progressive telomere shortening with each cell division is observed.

Telomerase has been shown to remain active in proliferative tissues such as bone marrow, skin, gastrointestinal tract, liver, testis and activated lymph nodes (Burger *et al.*

1997; Yui *et al.* 1998; Bachor *et al.* 1999; Liu *et al.* 1999; Forsyth *et al.* 2002). Of these tissues it has been observed that telomerase activity is highest in the stem and progenitor cell compartments, such as early haematopoietic progenitor cells, and is increased with proliferation and differentiation of these cells (Yui *et al.* 1998). However, these cells do exhibit progressive telomere shortening and it is thought that although expression of telomerase activity reduces telomere shortening it is not efficient to prevent telomere shortening as a result of proliferation (Engelhardt *et al.* 1997).

### ***1.5 Telomeres and telomerase in cancer***

Telomere shortening was first implicated in human cancer in 1990. Telomeres can be measured by first digesting genomic DNA with frequent cutting restriction endonucleases, such as HinfI, that do not cut telomeric DNA. The digested DNA is then subjected to southern blotting and telomere repeats are detected with a telomere specific probe. Hastie *et al.* showed that in patients with colorectal carcinomas telomere length was reduced when compared to normal colonic mucosa from the same patient (Hastie *et al.* 1990). While de Lange *et al.* demonstrated that in two Wilms' tumour samples, DNA from the tumour had significantly shorter telomeres than DNA from the surrounding normal kidney tissue and that telomere length was reduced in breast carcinoma when compared with normal breast tissue in the same patient (de Lange *et al.* 1990). These initial findings lead to extensive investigation into the role of telomere shortening in human cancer and since this initial work shortened telomeres have now been detected in the majority of human cancers (Blasco 2005). Not only are shorter telomeres detected in cancer when compared to normal tissues but telomeres shorten progressively as disease progresses. For example, in hepatocellular carcinoma progressive telomere shortening is observed as the disease progresses from normal to

chronic active hepatitis to hepatocellular carcinoma (Miura *et al.* 1997). Progressive telomere shortening is particularly well demonstrated in the leukaemias where serial blood samples can easily be obtained. For example in Chronic Myeloid Leukaemia telomere length in advanced disease stage is reduced when compared to chronic phase disease (Boulwood *et al.* 1999). Additionally, shorter telomeres at diagnosis often correlate with reduced survival in cancer and this has been demonstrated in many cancers including B cell chronic lymphocytic leukaemia, prostate cancer and lung cancer (Bechter *et al.* 1998; Donaldson *et al.* 1999; Frias *et al.* 2008). However, this is not always the case, and in tumours where patients have significantly shorter telomeres than normal tissues patients with longer telomeres relative to other cancer samples are predictive of reduced survival. For example, in colorectal and head and neck cancer those patients with longest telomeres have poor disease free survival (Patel *et al.* 2002; Garcia-Aranda *et al.* 2006). In other tumours the prognostic value of telomere length is not always clear. Short telomeres in neuroblastoma have been reported to correlate with both favourable and unfavourable prognosis (Hiyama *et al.* 1992; Ohali *et al.* 2006). Similarly in breast cancer some authors report no significant association with telomere length and disease prognosis while others suggest a correlation between shorter telomeres and poor prognosis (Griffith *et al.* 1999; Lu *et al.* 2011).

The first association of increased telomerase activity with human cancer came in 1994 with the development of the TRAP assay by Kim *et al.* (Kim *et al.* 1994). They demonstrated that telomerase activity was not present in normal somatic tissues or cultured cells. They showed that telomerase appeared to be reactivated in cancer biopsies and immortal cultured cells. Since then telomerase activity has been detected in at least 90% of human tumours (Kim *et al.* 1994; Shay and Bacchetti 1997). The detection of telomerase activity has been used as an important predictor of poor clinical

outcome in many different cancers. For example, in neuroblastoma, telomerase activity is not detected in normal adrenal tissue or in benign ganglioneuromas but is detected in almost all cancer samples. Additionally, those patients with highest telomerase activity exhibited additional genetic changes and had an unfavourable prognosis (Hiyama *et al.* 1995). Similarly in colorectal cancer high telomerase activity levels are associated with significantly poor prognosis and reduced disease free survival when compared with patients with low or moderate telomerase activity (Tatsumoto *et al.* 2000). Telomeres in colon cancer are reported to be longer in patients with advanced disease and high telomerase activity than those with early stage disease and low telomerase activity leading the authors to suggest that telomerase has been up-regulated in order to stabilise telomeres in these patients (Engelhardt *et al.* 1997). A correlation of increased telomerase activity and poor disease prognosis has been presented in many other cancers including lung cancer and acute leukaemia (Ohyashiki *et al.* 1997; Marchetti *et al.* 1999). However, this is not always the case and in cervical cancer telomerase activity is detected in 79% of samples but it has been reported that telomerase activity measurements do not correlate with disease stage, histotype, metastasis or overall disease prognosis (Wisman *et al.* 2001). This implies that presence of telomerase activity can distinguish normal from neoplastic cells but does not always predict disease outcome.

Telomerase activity can be employed as an additional screen for diagnosis of cancer. In bladder cancer early stage tumours can be missed due to the lack of effective cytological markers for detection. However, analysis of bladder washings revealed that telomerase activity can be detected in up to 90% of bladder cancers in bladder washings (Kinoshita *et al.* 1997). The authors show that measuring telomerase activity is more sensitive for cancer detection than cytologic analysis alone and using these methods in combination

is important for early cancer diagnosis in these cases. Similar results have been obtained in breast cancer. The measurement of telomerase activity in fine needle aspirates of breast cancer patients has improved diagnosis rates to 86% from 70% using cytology alone (Hiyama *et al.* 2000). The authors suggest that the technique is especially useful in detecting false negative results from cytological samples and increases diagnostic accuracy.

Telomerase levels and telomere length have been investigated in haematological malignancies. In patients with Myelodysplastic Syndromes (MDS) expression of *hTERT* and telomerase activity is increased in bone marrow of patients and those patients with highest telomerase have worst prognosis (Fu and Chen 2002; Briatore *et al.* 2009). Telomere length in MDS is variable but shortened telomeres are related to complex karyotypic abnormalities which indicates genomic instability and poor prognosis (Ohyashiki *et al.* 1994; Boulton *et al.* 1997). In Acute Myeloid Leukaemia (AML) telomere length is significantly decreased and higher telomerase activity is associated with higher rates of complete remission (Seol *et al.* 1998; Hartmann *et al.* 2005). Telomerase levels are heterogeneous in lymphoma but in general telomerase is increased in lymphoma and telomeres are shortened (Davison 2007).

Telomerase activity is increased in the majority of cancer while telomere length is decreased. This apparent paradox is explained by tumours having multiplied to such an extent that telomerase activation is essential for cell survival to stabilise telomere length and halt the loss of further telomeric DNA (Counter *et al.* 1992).

### ***1.6 Alternative Lengthening of Telomeres***

Although telomerase activity has been detected in the majority of human cancers there remain some 10% without detectable telomerase activity. These cancers maintain telomere length without telomerase activity using an Alternative Lengthening of Telomeres (ALT) mechanism. Telomeres of telomerase negative immortal human cells can be maintained by ALT and this gives rise to very long or short heterogeneous telomeres (Bryan *et al.* 1995). The mechanism of this telomere maintenance has been linked with recombination as individual telomere lengths can increase or decrease rapidly in these cells (Murnane *et al.* 1994). Furthermore, it has been demonstrated that telomeres in ALT cells can copy telomere sequences from one telomere to another but this does not occur in telomerase positive cells (Dunham *et al.* 2000). Additionally, telomere mutations observed in ALT cells are thought to be propagated by recombination and contribute to genomic instability (Varley *et al.* 2002).

Cells that maintain telomeres through ALT exhibit some distinct characteristics from normal telomerase positive cells. Extrachromosomal circular DNA fragments containing telomeric DNA have been observed in ALT nuclei and it is suggested that these are caused by homologous recombination (Cesare and Griffith 2004; Wang *et al.* 2004). Promyelocytic leukaemia (PML) nuclear bodies are characteristic of ALT cells and these PML bodies contain PML protein, telomeric DNA sequences and the telomere binding proteins TRF1 and TRF2 (Yeager *et al.* 1999). Although the function of ALT PML bodies remains unclear they also contain proteins that are involved in recombination and are required for ALT so it has been suggested that they are the site for ALT activity (Yeager *et al.* 1999).



ALT can be detected using combined immunofluorescence for PML and telomere fluorescence in situ hybridisation. This approach has been employed to demonstrate that ALT is acting in a substantial proportion of soft tissue sarcomas, osteosarcomas and astrocycomas (Henson *et al.* 2005). Gene expression profiling of cell lines has revealed a likely mesenchymal stem cell origin for tumours maintaining telomeres by ALT (Lafferty-Whyte *et al.* 2009). In fact, ALT is detected in carcinomas of mesenchymal stem cell origin such as liposarcoma, glioblastoma multiforme and peritoneal mesothelioma (Hakin-Smith *et al.* 2003; Johnson *et al.* 2005; Villa *et al.* 2008). ALT has also been detected in a small percentage of breast carcinomas that overexpress HER-2 by genomic amplification and the authors suggest a common mechanism for ALT and HER-2 overexpression (Subhawong *et al.* 2009).

### ***1.7 Telomerase inhibitors***

The association of increased telomerase activity with cancer has led to the suggestion that inhibiting telomerase in malignancies would reduce telomerase activity and telomere length to a level that would induce apoptosis in cancer cells resulting in a novel treatment option for many malignancies (Shay and Wright 2002). Several approaches to targeting telomerase have been investigated as anti-cancer therapies. These include agents to directly inhibit the telomerase enzyme such as small molecule oligonucleotides against hTERT or hTR and immunotherapy against hTERT. The most clinically advanced of these approaches in terms of clinical trials is immunotherapy against hTERT. Immunotherapy using peptides corresponding to regions of the hTERT protein relies on the patients potential to mount an immune response to the peptide. It has been shown that when immunised with a telomerase peptide most cancer patients subsequently have circulating T lymphocytes specific for the peptide, however, not all

of these patients produce cytotoxic T lymphocytes that have the ability to kill telomerase positive tumour cells (Filaci *et al.* 2006). The hTERT peptide vaccine GV1001 has been tested in clinical trials of patients with inoperable pancreatic cancer. Results from non randomised phase I and II clinical trials revealed that patients could mount an immune response and that the immune response could be correlated with increased survival (Bernhardt *et al.* 2006). A phase III study of GV1001 was then carried out with the aim of recruiting 520 patients with inoperable and metastatic pancreatic cancer. However, the study was halted after 365 patients had been enrolled when it was shown that treatment with GV1001 conferred no survival benefit (Buanes and Nemunaitis 2009). A further phase III study of GV1001 in patients with advanced or metastatic pancreatic cancer is recruiting at present with estimated enrolment of 1110 patients (clinicaltrials.gov identifier NCT00425360). The primary end point of this study would be survival at one year.

Inhibition of telomerase by small molecules and oligonucleotides has also been investigated for therapeutic use. One example of this is GRN163L which is a complimentary hTR oligonucleotide that directly binds hTR so that the RNA template is not accessible and this causes a reduction of telomerase activity (Dikmen *et al.* 2005). Initial investigation of this compound in telomerase positive lung cancer cells showed a decrease in telomerase activity and prevention of lung cancer metastases in animal models (Dikmen *et al.* 2005). Currently GRN163L is involved in thirteen on-going clinical trials including randomised phase II trials for breast cancer and non-small cell lung cancer (Genron Corporation). Results of these trials are not yet available. Results from a phase I trial in advanced solid tumours indicates that the drug causes thrombocytopenia at high doses but does not result in any other major toxicities leading

to GRN163L to be anticipated as a universal cancer therapeutic with minimal side effects (Shay and Wright 2006; Ratain *et al.* 2008).

Telomerase inhibitors are an attractive treatment option for the majority of cancers that express telomerase. However, it has been suggested that telomerase inhibitors might result in drug resistant telomerase independent cancer cells emerging. ALT mechanisms could be activated by the inhibition of telomerase or the telomerase inhibitors may select for those cells that maintain telomeres using ALT (Keith *et al.* 2002).

### ***1.8 Aims of the study***

Telomerase regulation and telomere length clearly play an important role in the progression of the majority of human cancers. This study aims to gain further insight into the role of telomerase regulation and expression in the pathogenesis of CML. Real time PCR has been used to quantify gene expression of *hTERT*, the catalytic component of telomerase, its accessory components (*hTR* and *TEP1*) its regulators (*c-Myc* and *PinX1*) and the telomere associated proteins *TRF1*, *TRF2* and *tankyrase* in CML. Regulation of *hTERT* was further investigated by analysis of promoter methylation and mutation status. The *BCR/ABL* translocation was monitored by real time PCR to identify any correlation with levels of telomerase associated genes. cDNA microarray technology was used to analyse gene expression in the neutrophils of patients with Essential Thrombocythaemia harbouring the *JAK2* V617F mutation which, like the *BCR/ABL* translocation in CML, results in an activated kinase. Affymetrix gene expression analysis was used to investigate the role of telomerase related genes in different subtypes of MDS. The identification of aberrant regulation of telomerase and

associated genes may provide novel insights into the role of telomerase and telomeres in the pathogenesis of these diseases.

## ***Chapter 2***

### ***Materials and Methods***

## ***2.1 Preparation of Granulocyte Fractions from Peripheral Blood***

Forty milliliters of peripheral blood was routinely collected and granulocyte fractions were separated in two batches of 20 ml. The method below describes separation of 40 ml peripheral blood.

### ***2.1.1 Separation of Granulocytes by Density Gradient Centrifugation***

1. 40 ml of peripheral blood was collected into Tri-Sodium EDTA tubes.
2. The blood was separated into two aliquots of 20 ml and each aliquot was gently layered onto 20 ml of room temperature Histopaque-1077 (Sigma Aldrich, Cheshire, UK) in a 50 ml polypropylene centrifuge tube and centrifuged at 1,600 rpm (400g, Sorvall RT6000B benchtop centrifuge) for 30 minutes at room temperature.
3. The serum and Histopaque layers were carefully aspirated using a Pasteur pipette and discarded to leave the red blood cell/granulocyte layers.
4. The red blood cell/granulocyte layers were washed by adding Ice-cold Phosphate Buffered Saline (PBS) containing 5 mM EDTA to a total volume of 50 ml and the tubes were mixed by gentle inversion.
5. The tubes were centrifuged at 1,650 rpm at 4°C for 10 min.
6. The supernatant was removed and discarded using a Pasteur pipette.
7. Wash steps 4, 5 and 6 were repeated.
8. To lyse the red cells, the packed red blood cell/granulocyte layer was distributed into centrifuge tubes containing freshly prepared ice-cold red cells lysis buffer (0.1mM EDTA, 150mM  $\text{NH}_4\text{Cl}$ , 10mM  $\text{NaHCO}_3$ ). Approximately 2 ml of red blood cells were added to each 50 ml of red cell lysis buffer and left on ice for 15 min with occasional mixing.

10. The tubes were centrifuged at 1,650 rpm for 10 min at 4°C and the supernatant was poured off and discarded.
11. Each granulocyte pellet was resuspended in 1 ml of ice-cold PBS and the pellets were pooled into two centrifuge tubes.
12. The tubes were filled to 50 ml with ice-cold PBS and centrifuged at 1,600 rpm for 10 min at 4°C. The supernatant was poured off and discarded.
13. The granulocyte pellets were resuspended in 1 ml of ice-cold PBS and pooled into one centrifuge tube.
14. The tube was filled to 50 ml with ice-cold PBS.
15. An aliquot of the granulocyte suspension was taken and serial dilutions were prepared.
16. Cytocentrifuge preparations were made in a Shandon Cytospin 2 using 100 µl of granulocyte suspension and one drop of 22% Bovine Serum Albumin (BSA) per slide. Samples were spun for 5 min at 800 rpm.
17. The centrifuge tube was centrifuged at 1,600 rpm for 10 min at 4°C. The supernatant was poured off and discarded.
18. The granulocyte pellet was then resuspended in TRIzol reagent (Invitrogen, Paisley, UK) as described in section 2.4.

### ***2.1.2 Wright-Giemsa Staining of Cytocentrifuge Preparations***

Granulocyte cytocentrifuge slides were stained with Wright-Giemsa staining solution in a Miles Hema-Tek 2000 automated slide stainer. The slides were subjected to the following steps:

1. Wright-Giemsa stain (Wright-Giemsa stain 0.15% in methanol)

2. Rinse solution 1 (PBS)
3. Rinse solution 2 (Ethanol 18% v/v with surfactant)
4. Air drying

The stained cytocentrifuge slides were then inspected to determine the purity of the granulocyte preparations.

## ***2.2 Preparation of CD34<sup>+</sup> progenitor cells from bone marrow samples***

CD34<sup>+</sup> progenitor cells were isolated from bone marrow samples using the CD34 MicroBead Kit from Miltenyi Biotec (Bergisch Gladbach, Germany).

### ***2.2.1 Isolation Strategy***

The CD34 microbead kit allows positive selection of CD34 expressing cells using MACS MicroBeads conjugated to mouse anti-human CD34 antibody. Bone marrow samples were separated by density gradient centrifugation over Histopaque-1077 to isolate the mononuclear cells. CD34<sup>+</sup> haematopoietic progenitor cells were then magnetically labelled with the CD34 MicroBeads and enriched on positive separation columns in the magnetic field of a MidiMACS system. This kit allows isolation of haematopoietic progenitor cells which make up 0.5 – 3% of total bone marrow cells to be enriched to a purity of 90-98%.



### ***2.2.2 Preparation of Bone Marrow Cells***

1. Bone marrow samples were obtained using a heparinised syringe and collected in a sterile 50 ml tube containing 5 ml PBS supplemented with 2mM EDTA.
2. The sample was diluted in 50 ml RPMI-1640 medium with 10 mg of DNase I and 10 mg of Collagenase B in order to dissociate the cells.
3. The sample was incubated for 45 min in a 37°C water bath and mixed occasionally.
4. The cell suspension was passed through a 70 µm cell strainer.
5. The cell suspension was carefully layered over Histopaque-1077 (approximately 25 ml of cell suspension over 15 ml of Histopaque-1077) in sterile 50 ml centrifuge tubes.
6. The tubes were centrifuged at 1,600 rpm for 30 min at room temperature.
7. The upper layer was carefully removed leaving the mononuclear cell layer undisturbed at the interphase.
8. The interphase mononuclear cells were collected and transferred to a new 50 ml centrifuge tube, Hanks' Balanced Salt solution (HBSS) was added to a total volume of 50 ml and the tube was mixed by gentle inversion.
9. The tube was centrifuged at 1,450 rpm for 12 min at room temperature and the supernatant was poured off.
10. The mononuclear cell pellet was resuspended in 1 ml of HBSS.
11. The tube was filled to 50 ml with HBSS and centrifuged at 1,450 rpm for 12 min at room temperature. The supernatant was discarded.
12. The mononuclear cell pellet was resuspended in 25 ml of MACS buffer (PBS + 0.5% BSA). A 10 µl aliquot was taken and cells were counted in a haemocytometer.

13. The tube was centrifuged at 1,300 rpm for 10 min at room temperature and the supernatant was discarded.
14. The mononuclear cell pellet was resuspended in 300  $\mu$ l of MACS buffer (the volume of MACS buffer was reduced by half if mononuclear cells were  $< 5 \times 10^7$ , or doubled if mononuclear cells were  $> 8 \times 10^8$ ).

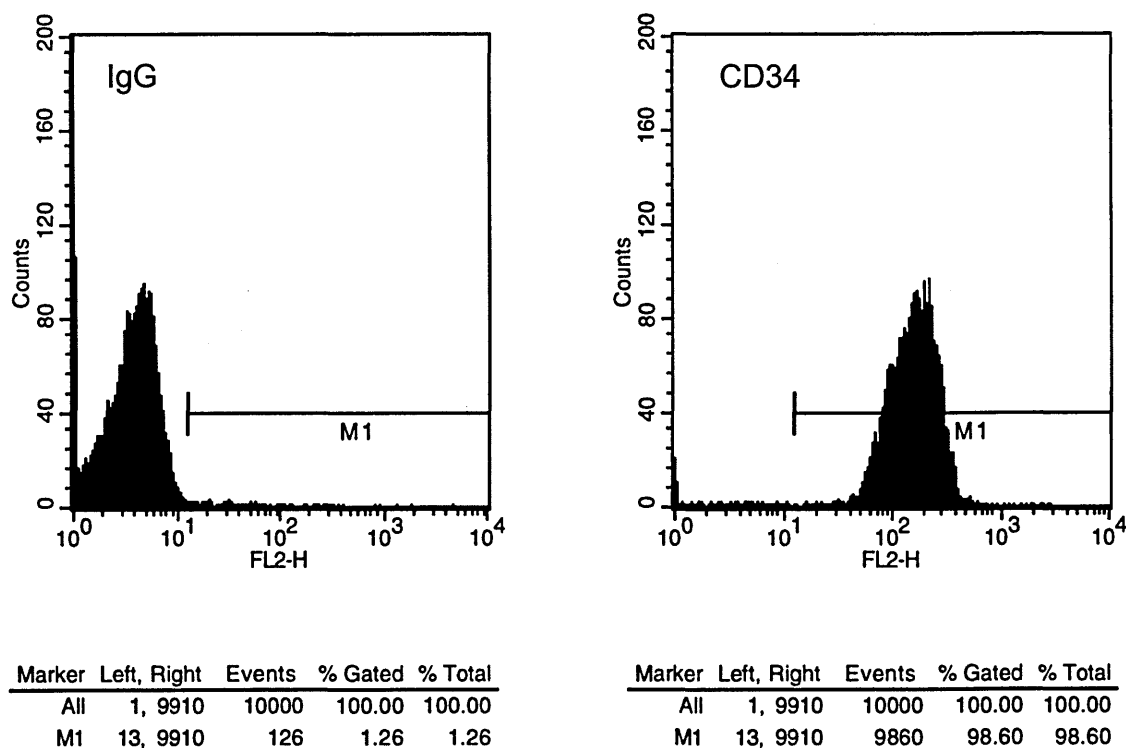
### ***2.2.3 Magnetic Labelling of CD34<sup>+</sup> Progenitor Cells***

1. 100  $\mu$ l of FcR Blocking Reagent was added to the cell suspension to inhibit unspecific binding of the CD34 MicroBeads to Fc-receptors on other cells. If necessary the volume was adjusted according to cell number.
2. Cells were labelled by adding 100  $\mu$ l of CD34 Microbeads. Again, if necessary, the volume was adjusted according to cell number. Samples were then mixed gently and incubated for 30 min at 4°C.
3. The tube was filled with MACS buffer to 25 ml and centrifuged at 1,300 rpm for 5 min at room temperature. The supernatant was discarded.
4. The cell pellet was resuspended in 1 ml of MACS buffer.

### ***2.2.4 Magnetic Separation of Mononuclear Cells***

1. A magnetic MidiMACS Separation Unit was attached to a MACS MultiStand.
2. One LS Separation Column was placed in the magnetic field of the MidiMACS Separation Unit and a collection tube was placed under the column.
3. The column was prepared by rinsing with 3 ml of MACS buffer.
4. The magnetically labelled cell suspension was passed through a 30- $\mu$ m nylon pre-separation filter to remove cell clumps.

5. The cells were added to the column and allowed to pass through.
6. The column was washed three times with 1 ml of MACS buffer.
7. The column was removed from the separator and placed in a new collection tube.
8. 3 ml of MACS buffer was added to the column and the magnetically labelled cells were firmly flushed out using the plunger supplied with the column.
9. The magnetic separation was repeated with a second LS Separation Column to increase the purity of the positive fraction and the cells were flushed out in a new collection tube with 2.5 ml of MACS buffer.
10. A 10  $\mu$ l aliquot of cells was taken and the cells were counted in a haemocytometer.
11. Two aliquots of  $5 \times 10^4$  cells were taken for evaluation of CD34<sup>+</sup> cell purity by flow cytometry (see section 2.2.5).
12. The sample was centrifuged at 1,450 rpm for 10 min at room temperature and the supernatant was carefully removed.
13. The cell pellet was resuspended in TRIzol reagent as described in section 2.4.



**Figure 2.1 Determination of CD34<sup>+</sup> cell purity by flow cytometry.** Gate M1 was used to identify levels of nonspecific binding in the IgG1 labelled sample. This was then superimposed to the CD34 labelled sample and the number of positive cells calculated.

### 2.2.5 Evaluation of CD34<sup>+</sup> Cell Purity by Flow Cytometry

1. The two aliquots of  $5 \times 10^4$  eluted cells were transferred to flow cytometry tubes and the volume adjusted to 45  $\mu$ l with MACS buffer.
2. 5  $\mu$ l of PE-conjugated anti-mouse IgG1 antibody was added to the first aliquot of cells to correspond to the negative control; while 5  $\mu$ l of PE-conjugated anti-CD34 antibody was added to the second aliquot to label the positive cells.
3. The samples were mixed gently and incubated at 4°C for at least 30 min.
4. CD34<sup>+</sup> cell purity was determined by loading the samples into a FACScalibur flow cytometer and acquiring 10,000 events for both negative and positive samples

(Figure 2.1). The positive and negative plots were examined using cellquest software. The negative sample shows the levels of non-specific binding of the antibodies to the cells.

### ***2.3 Preparation of Platelet Fractions from Peripheral Blood***

Platelets were isolated from whole blood from patient or normal control samples using the method described by Wang *et al.* (Wang *et al.* 2003). Platelet purity was assessed by flow cytometry.

#### ***2.3.1 Separation of Platelets***

1. 50 ml of peripheral blood was collected into Tri-Sodium EDTA tubes.
2. The blood was transferred to a 50 ml centrifuge tube and a 2  $\mu$ l aliquot of sample was taken for flow cytometry.
3. The tube was centrifuged at 150 g for 20 min.
4. The top 75% of the platelet rich plasma layer was transferred carefully to a fresh tube. A 5  $\mu$ l aliquot of sample was taken for flow cytometry.
5. The plasma was centrifuged at 150 g for 10 min.
6. The plasma was removed to a fresh tube and any pelleted cells were discarded. A 5  $\mu$ l aliquot of sample was taken for flow cytometry.
7. The platelets were pelleted by centrifugation at 800 g for 15 min and the supernatant was discarded.
8. The pellet was washed in 10 ml of PBS and a 5  $\mu$ l aliquot of sample was taken for flow cytometry.

9. The platelets were pelleted by centrifugation at 800 g for 15 min and the supernatant was discarded. The pellet was resuspended in TRIzol reagent as described in section 2.4.

### ***2.3.2 Evaluation of Platelet Purity by Flow Cytometry***

1. The aliquots of cells removed for flow cytometry were each added to a separate flow cytometry tube.
2. 5 µl of each of the following antibodies were added to each tube:  
FITC-conjugated anti-human CD41 to label platelets (Dako, Glostrup, Denmark)  
PerCP-conjugated anti-human CD45 to label leukocytes (BD Biosciences, San Jose, USA)  
PE-conjugated anti-human Glycophorin A to label red blood cells (BD Biosciences).
3. Pharmingen Stain Buffer (BSA) (BD Biosciences) was added to each sample to a final volume of 50 µl.
4. The samples were incubated at room temperature for 30 min then 950 µl of Pharmingen Stain Buffer (BSA) was added to each sample.
5. The samples were loaded on to the FACScalibur flow cytometer.

## ***2.4 Extraction of nucleic acids from cell preparations***

### ***2.4.1 Nucleic Acid Extraction using TRIZOL Reagent***

Total RNA and genomic DNA were extracted from pre separated cell preparations using the TRIZOL reagent from Invitrogen (Paisley, UK).

1. 1 ml of TRIZOL reagent was added per  $10^7$  cells and the cells were lysed by vortexing in a 15 ml tube.
2. The lysed sample was incubated at room temperature for 5 min. The lysate was then split into 1.25 ml aliquots in 1.5 ml microcentrifuge tubes.

#### ***2.4.1.1 RNA isolation***

3. 0.25 ml of chloroform was added per 1.25 ml of TRIZOL reagent used.
4. The samples were mixed vigorously for 15 sec and incubated at room temperature for 2-3 min.
5. The samples were centrifuged at 12,000 g for 15 min at 4°C.
6. The aqueous upper phase was transferred to a new 1.5 ml microcentrifuge tube and the interphase and phenol phase were set aside for subsequent DNA extraction.
7. 0.625 ml of Isopropanol per 1.25 ml TRIZOL reagent used was added to the aqueous phase and mixed by inversion.
8. The samples were incubated for 10 min at room temperature and then centrifuged at 12,000 g for 15 min at 4°C. The RNA precipitate could then be visualised as a pellet at the bottom of the tube.
9. The supernatant was removed carefully and the pellet was washed with 75% ethanol adding 1.25 ml per 1.25 ml TRIZOL reagent used. The sample was vortexed and centrifuged at 10,000 g for 5 min at 4°C.
10. The supernatant was removed and the pellet was air-dried.
11. The pellet was resuspended in 50-100  $\mu$ l DEPC H<sub>2</sub>O and incubated at 60°C until the pellet had completely dissolved. The samples were stored at -70°C.

#### ***2.4.1.2 DNA Isolation***

1. 0.375 ml of 100% ethanol was added to the interphase and phenol phase from the RNA isolation.
2. The samples were mixed by inversion and incubated for 2-3 min at room temperature then centrifuged at 10,000 g for 5 min at 4°C.
3. A pellet was then visible and the supernatant was carefully removed.
4. 1.25 ml of 0.1 M Sodium Citrate in 10% Ethanol was added to the pellet and the samples were incubated at room temperature for 30 min.
5. The samples were centrifuged at 10,000 g for 5 min at 4°C and the supernatant was removed.
6. Steps 4 and 5 were repeated.
7. The pellets were washed in 1.4 ml of 75% ethanol and incubated for 10-20 min at room temperature. The pellets were centrifuged at 10,000 g for 5 min at 4°C.
8. The supernatant was removed and the pellets were air dried then resuspended in molecular grade H<sub>2</sub>O. DNA was stored at -20°C.

#### ***2.4.2 DNA Extraction using the Qiagen QIAamp DNA Blood Maxi Kit***

The Qiagen QIAamp DNA blood maxi kit allows for the rapid purification of total DNA from whole blood and related body fluids using a spin column procedure.

1. 5 ml of peripheral blood was collected in Tri-Sodium EDTA tubes.
2. The blood was added to a 50 ml centrifuge tube containing 500 µl of QIAGEN Protease and mixed briefly.



3. 12 ml of Buffer AL was added and the tube mixed thoroughly by inverting 15 times. The tube was then shaken vigorously for 1 min.
4. The sample was incubated at 70°C for 10 min in a waterbath.
5. 10 ml 100% ethanol was added to the sample and the tube was inverted 10 times then shaken vigorously.
6. Half of the solution from step 5 was transferred to the QIAamp Maxi column placed in a 50 ml centrifuge tube then centrifuged at 3000 rpm for 3 min.
7. The QIAamp Maxi column was removed and the flow through discarded. The QIAamp Maxi column was then placed back into the 50 ml centrifuge tube and the remainder of the solution from step 5 added to the column and centrifuged at 3000 rpm for 3 min.
8. The QIAamp Maxi column was removed and the flow through discarded. The QIAamp Maxi column was then placed back into the 50 ml centrifuge tube and 5 ml of Buffer AW1 was added to the column and centrifuged at 5000 rpm for 1 min.
9. 5 ml of Buffer AW2 was added to the QIAamp Maxi column and centrifuged at 5000 rpm for 15 min.
10. The QIAamp Maxi column was placed in a clean 50 ml centrifuge tube and the collection tube containing the flow through was discarded
11. 1 ml of molecular grade H<sub>2</sub>O equilibrated to room temperature (15–25°C) was added directly onto the membrane of the QIAamp Maxi column and incubated at room temperature for 5 min then centrifuged at 5000 rpm for 2 min.
12. The eluate containing the DNA was then reloaded onto the membrane of the QIAamp Maxi column and incubated at room temperature for 5 min then centrifuged at 5000 rpm for 5 min. The DNA was stored in aliquots of 1 ml at -20°C.

## ***2.5 The Polymerase Chain Reaction (PCR)***

Thermo-Start Hot-start Taq DNA polymerase (Abgene, UK) was used to generate specific PCR products for the DNA sequences of interest. The hot-start enzyme remains inactive until it is activated at high temperature. Primers were designed to be specific to the appropriate sequence and dissolved at a concentration of 100 pmol/ $\mu$ l in Nuclease-Free H<sub>2</sub>O and stored at -20°C. Each primer set was optimised using control DNA samples by performing PCR at different annealing temperatures to determine the optimal reaction conditions.

### ***2.5.1 PCR using Thermo-Start DNA Polymerase***

A ready made 2x Thermo-Start PCR Master Mix was used. The final 1x reaction mixture contained the following components: 1.5 units Thermo-Start DNA Polymerase, 1x Thermo-Start buffer, 1.5 mM MgCl<sub>2</sub> and 0.2 mM dNTP mix.

1. A PCR master mix was prepared for each primer set for the required number of samples plus a negative control. The volumes of reagents for one sample were as follows:

2x Thermo-Start PCR Master Mix	25 $\mu$ l
Forward primer	1.25 $\mu$ l
Reverse primer	1.25 $\mu$ l
Nuclease-Free H <sub>2</sub> O	21.5 $\mu$ l

2. 49  $\mu$ l aliquots of the PCR master mix were added to individual wells of a 96 well PCR plate and 1  $\mu$ l of DNA template was added to the appropriate wells.

3. The plate was centrifuged briefly.

4. The PCR amplification was performed using the 9700 PE GeneAmp PCR System (Applied Biosystems, UK) using the thermal cycling profile detailed in Table 2.1

**Table 2.1 Thermal cycling profile for ThermoStart DNA polymerase**

	Temperature	Time	Number of cycles
Enzyme activation	95°C	15 min	1 cycle
Denaturation	95°C	10 sec	35 cycles
Annealing	50-65°C	30 sec	
Extension	72°C	0.5-1 min	
Final extension	72°C	5 min	1 cycle
Hold temperature	4°C	∞	1 cycle

### **2.5.2 Agarose Gel Electrophoresis of PCR Products**

Agarose gel electrophoresis was used to resolve and visualise PCR products. 1% (w/v) agarose gels were typically used.

1. The required amount of agarose was added to 1x TBE (130 mM Tris, 45 mM Boric Acid, 2.5 mM EDTA) and heated in a microwave to dissolve the agarose.
2. The gel solution was allowed to cool at room temperature for approximately 5 min and ethidium bromide was added at a dilution of 1 in 10,000.
3. The gel solution was poured into a gel tray and combs were inserted. The gel was left to set at room temperature.
4. 5 µl of each PCR product was mixed with 1 µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% Glycerol).
5. The set gel was placed in a gel tank and submerged in 1 x TBE. 5 µl of Hyperladder IV (Bioline, UK) was added to the first well.

6. The PCR products were added to subsequent wells and the gel was run at 5 V/cm for approximately 40 min.
7. The bands were visualised by placing the gel in a UV transilluminator.
8. The remaining PCR products were stored at -20°C in the 96 well plate until required for further analysis.

### ***2.5.3 PCR Product Quantitation using the Agilent Bioanalyzer***

The Agilent Bioanalyser and the DNA 1000 kit were used to obtain accurate size and quantitative data of multiplex PCR products. It is a microfluidics based system that allows high resolution fragment analysis as low as 5 bp. Traditional gel electrophoresis principles have been transferred to a chip format and samples run along glass micro channels. The samples are mixed with an intercalating dye and detected by laser induced fluorescence. A DNA ladder of known size is run at the same time as the PCR products and each sample is run with two internal standard markers to align the PCR product data with the DNA ladder. The data can be plotted as a gel like image or an electropherogram plot of fluorescence intensity versus size.

1. The DNA dye concentrate and DNA gel matrix were equilibrated to room temperature for 30 min.
2. The DNA dye concentrate was vortexed and briefly centrifuged then 25 µl was added to the DNA gel matrix vial.
3. The gel dye mix was vortexed and transferred to a spin filter and centrifuged for 15 min at 6000 rpm.
4. The spin filter was discarded and 9 µl of gel dye mix was added to the well marked with a circled letter G on a new DNA chip in the chip priming station.

5. The chip was pressurised by depressing the plunger of the syringe attached to the chip priming station and the plunger was secured for 1 min.
6. After 5 sec the plunger was pulled up.
7. 9 µl of gel dye mix was added to the wells marked G.
8. 5 µl of DNA marker was added to the remaining wells.
9. 1 µl of DNA ladder was added to the well marked with the ladder symbol.
10. 1 µl of each sample to be analysed was added to the appropriate sample wells.
11. The chip was vortexed for 1 min at 2400rpm in the IKA vortex mixer.
12. The chip was loaded into the Bioanalyser instrument and the Agilent 2100 expert software was used to start the run for a DNA chip.
13. Electropherograms were viewed using the Agilent 2100 expert software and a run was considered successful when all 13 peaks of the DNA ladder were well resolved and both markers were correctly identified in each sample.

## ***2.6 Cycle Sequencing***

Cycle sequencing for mutation analysis of candidate genes was carried out using the ABI PRISM 3100 Genetic Analyser. Dye terminator chemistry was used and a 50cm ABI Genetic Analyser 16 Capillary Array and Performance Optimized Polymer 6 (POP-6) were required to run the samples.

### ***2.6.1 Purification of PCR Products using ExoSAP-IT***

The ExoSAP-IT reagent (USB) was used to purify PCR products prior to sequencing to remove contaminating primer dimers and unincorporated dNTPs that could interfere

with the sequencing reactions. ExoSAP-IT consists of the enzymes Exonuclease I and Shrimp Alkaline Phosphatase and cleans up PCR products without the loss of sample.

1. 5  $\mu$ l aliquots of each of the PCR products to be purified were added to a separate labelled 0.5 ml microcentrifuge tube.
2. 2  $\mu$ l of ExoSAP-IT was added to each tube on ice and tubes were mixed and centrifuged briefly.
3. The samples were incubated on a thermal cycler for 15 min at 37°C.
4. The samples were then incubated for 15 min at 80°C to deactivate the ExoSAP-IT.
5. The contents of the tubes were centrifuged briefly and 1 $\mu$ l aliquots were added immediately to prepared sequence reaction tubes.

### ***2.6.2 Preparation of Sequencing Reactions***

The BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems UK, Warrington, Cheshire) was used to prepare the sequencing reactions. This kit used dye terminator chemistry in which a different coloured fluorescent dye is attached to each of the four ddNTPs. Two sequencing reactions were prepared for each template, one each for the forward and reverse primers.

1. A master mix was prepared for each primer for the appropriate number of samples. The volume of components was as follows:

Terminator Ready Reaction Mix	4 $\mu$ l
5x Sequencing Buffer	2 $\mu$ l
Primer (3.2 pmol/ $\mu$ l)	1 $\mu$ l

Nuclease-Free H<sub>2</sub>O

12 µl

2. The master mix was vortexed and centrifuged briefly.
3. 19 µl aliquots of master mix were added to labelled 0.2 ml microfuge tubes and 1 µl of the corresponding purified PCR product was added.
4. The tubes were mixed and centrifuged briefly.
5. The tubes were then placed on the GeneAmp 9700 Thermal Cycler (Applied Biosystems) and subjected 25 cycles of 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min followed by a hold at 4°C until ready for purification.
6. The sequence reaction products were stored at -20°C at this point if necessary.

### ***2.6.3 Sodium Acetate Precipitation of Sequencing Reactions***

1. 2 µl each of 3M sodium acetate (pH 4.6) and 500 mM EDTA were added to each sequencing reaction tube with 70 µl of 100% ethanol.
2. The tubes were vortexed briefly and incubated on ice for at least 30 min or up to 24 hr to precipitate.
3. The tubes were centrifuged at 16,000g for 30 min.
4. The supernatant was carefully removed taking care not to dislodge the pellet.
5. 100 µl of 70% ethanol was added to each pellet and the tubes were vortexed briefly then centrifuged for 5 min at 16,000g.
6. The supernatant was removed and the tubes were centrifuged for a further 5 min.
7. Any remaining supernatant was carefully removed.
8. The pellets were left to air dry for 20 min.
9. Each pellet was resuspended in 20 µl of Hi-Di Formamide (Applied Biosystems) then vortexed to resuspend the pellets before centrifuging briefly. The samples can be stored at -20°C at this stage if necessary.

11. The samples were denatured at 95°C for 2 min and placed on ice.
12. 10 µl aliquots of the precipitated sequence reaction products were added to the 96 well sequencing plate and the plate was loaded on the 3100 Genetic Analyser.

#### ***2.6.4 Sequence Data Analysis***

The sequence data was collected using the ABI PRISM Data collection software version 1.0.1 and was processed using the ABI PRISM DNA Sequencing Analysis Software version 3.7 NT. Sequences were compared to that of the NCBI database by pairwise analysis using Sequence Analysis software (Informagen).

#### ***2.7 Cloning of PCR Products***

The pGEM-T Easy Vector System II was used to clone PCR products where required. The vector in this system has been linearised with a single 3' terminal thymidine at both ends to allow insertion of PCR products with single base adenine overhangs. Colonies containing vectors where the PCR product has been inserted successfully are identified using blue/white screening of transformants on IPTG/X-Gal indicator plates. When a PCR product has been inserted in the vector it will disrupt the coding region of the  $\beta$ -galactosidase enzyme and colonies will appear white. If no insert is present then the gene will not be disrupted and colonies will appear blue.

##### ***2.7.1 Ligation***

1. Ligation reactions were set up in 0.5 ml tubes as described in table 2.2. Before each use the 2x rapid ligation buffer was vortexed vigorously.



2. The reactions were mixed by pipetting and incubated overnight at 4°C.

**Table 2.2 Components of ligation reactions using the pGEM-T Easy Vector System**

**II**

Reaction Component	Standard Reaction	Positive Control	Background Control
2 x Rapid Ligation Buffer, T4 DNA Ligase	5 µl	5 µl	5 µl
pGEM-T Easy Vector (50 ng)	1 µl	1 µl	1 µl
PCR product	1 µl	-	-
Control Insert DNA	-	2 µl	-
T4 DNA Ligase (3 Weiss units/µl)	1 µl	1 µl	1 µl
nuclease-free H <sub>2</sub> O to a final volume of	10 µl	10 µl	10 µl

**2.7.2 Transformation**

1. The tubes containing the ligation reactions were centrifuged briefly.
2. 2 µl of each ligation reaction was added to a 1.5 ml microcentrifuge tube on ice.
3. One tube of frozen JM109 High Efficiency Competent Cells per ligation was placed in an ice bath until just thawed then cells were mixed by gently flicking the tube.
4. 50 µl of cells were added to each ligation reaction tube.
5. Reactions were flicked to mix and incubated on ice for 20 min.
6. The cells were heat-shocked for 50 sec in a water bath at 42°C then immediately returned to ice for 2 min.
7. 950 µl room temperature SOC medium (2% Tryptone (pancreatic digest of casein) (w/v), 0.5% Yeast extract (w/v), 8.6 mM NaCl, 2.5 mM KCl, 20 mM MgSO<sub>4</sub>, 20 mM Glucose) was added to the tubes.
8. The samples were incubated for 1.5 hr at 37°C with shaking at 150 rpm.
9. 100 µl of each transformation culture was plated onto LB/ampicillin/IPTG/ X-Gal plates.

10. The plates were incubated overnight at 37°C.
11. White colonies containing inserts were identified by eye before proceeding with purification.

### ***2.7.3 Purification of Plasmid DNA using the Wizard Plus Minipreps DNA Purification System***

The Wizard Plus Minipreps DNA Purification System (Promega) allows for small scale purification of plasmid DNA using the Vac-Man Laboratory Vacuum Manifold.

1. Each colony to be selected was transferred into 5 ml of LB/ampicillin in a separate 50 ml centrifuge tube.
2. The tubes were incubated overnight at 37°C with shaking at 150 rpm.
3. The cells were pelleted by centrifugation at 10,000 g for 10 min and the supernatant was removed before blotting the tube upside-down on a paper towel to remove excess media.
4. The cell pellets were resuspended in 300 µl of Cell Resuspension Solution and transferred to a 1.5 ml microcentrifuge tube.
5. 300 µl of Cell Lysis Solution was added to each sample and mixed by inverting the tube 4 times.
6. 300 µl of Neutralization Solution was added to each sample and mixed by inverting the tube several times.
7. The tubes were centrifuged at 10,000 g in a microcentrifuge for 5 min to pellet the cell debris.
8. For each miniprep one Wizard Miniprep Column was prepared by attaching a Syringe Barrel to the Luer-Lok extension of each Minicolumn. The tip of the

Minicolumn/Syringe Barrel assembly was inserted into the vacuum manifold and all of the stopcocks closed.

9. 1 ml of resuspended Wizard Minipreps DNA Purification Resin was added to each barrel of the Minicolumn/syringe assembly.

10. The cleared lysate from each miniprep (supernatant from Step 7) was transferred to the barrel of the Minicolumn/syringe assembly containing the resin.

11. The stopcocks were opened and a vacuum was applied until all of the resin/lysate mix had been pulled into the Minicolumn.

12. 2 ml of Column Wash Solution was added to each Syringe Barrel and the vacuum applied.

13. The resin was dried by continuing to draw a vacuum for 30 seconds after the solution had been pulled through the column.

14. The Syringe Barrels were removed and the Minicolumns were transferred to 1.5ml microcentrifuge tubes.

15. The Minicolumns were centrifuged at 10,000 g in a microcentrifuge for 2 min to remove any residual Column Wash Solution.

16. The Minicolumns were transferred to a new microcentrifuge tube and 50  $\mu$ l of nuclease-free H<sub>2</sub>O was added to each Minicolumn and incubated for 1 min.

17. The tubes were centrifuged at 10,000 g in a microcentrifuge for 20 sec to elute the DNA.

18. The minicolumns were discarded and purified plasmids were stored at -20°C.

19. Plasmid sequences were confirmed by Cycle Sequencing.

## ***2.8 Bisulfite Modification of DNA for Methylation Specific PCR (MSP)***

The CpGenome DNA modification kit was used to bisulfite modify purified genomic DNA from patient and control samples. Using this process any unmethylated cytosines are deaminated and sulfonated which will convert them to Uracil. Any methylated cytosines are protected from bisulfite modification and remain unchanged.

### ***2.8.1 Preparation of Solutions***

1. 3 M NaOH stock was freshly prepared prior to each modification.
2. 20 mM NaOH/90% ethanol was freshly prepared prior to each modification.
3. For each sample to be modified 0.227 g of DNA Modification Reagent I was added to 0.571 ml of H<sub>2</sub>O and mixed by vortexing.
4. The pH of reagent I was adjusted to 5.0 with approximately 20 µl of 3M NaOH.
5. 1 µl of β-mercaptoethanol was added to 20 ml of deionized H<sub>2</sub>O and for each sample to be modified 750 µl of this solution was added to 1.35 g of DNA Modification Reagent II and mixed well.

### ***2.8.2 DNA Modification Procedure***

1. For each DNA sample to be modified 7 µl of 3M NaOH was added to 1 µg of DNA in 100 µl of water in a 2 ml screwcap microcentrifuge tube.
2. The DNA was incubated for 10 min at 50°C in a water bath.
3. 550 µl of DNA Modification Reagent I was added to each sample and vortexed.
4. The samples were incubated at 50°C for 16 hr in a water bath protected from light.

5. DNA Modification Reagent III was resuspended by vortexing and drawn into and out of a 1 ml pipette tip to disperse any remaining clumps.
6. 5  $\mu$ l of DNA Modification Reagent III was added to the DNA samples in the tubes.
7. 750  $\mu$ l of DNA Modification Reagent II was added to each sample and mixed briefly.
8. The samples were incubated at room temperature for 5-10 min.
9. The samples were centrifuged for 10 sec at 5,000 g to pellet the DNA Reagent III and supernatant was discarded.
10. 1 ml of 70% ethanol was added to each sample then samples were vortexed and centrifuged for 10 sec at 5,000 g and supernatant was discarded.
11. Step 10 was repeated two more times.
12. The samples were centrifuged at 13,000 g for 2 min the remaining supernatant was removed.
13. 50  $\mu$ l of the 20 mM NaOH/90% ethanol solution was added to the samples.
14. The samples were vortexed briefly to resuspend the pellet then incubated at room temperature for 5 min.
15. The samples were centrifuged for 10 sec at 5,000 g.
16. 1 ml of 90% ethanol was added and tubes were vortexed to wash the pellet. The samples were centrifuged for 10 sec at 5,000 g and the supernatant was removed.
17. Step 16 was repeated.
18. The tubes were centrifuged at 13,000 g for 3 min.
19. The pellets were allowed to air dry for 10-20 min at room temperature.
20. 20  $\mu$ l of TE Buffer was added to each sample and the tubes were vortexed.
21. The samples were incubated for 15 min at 56°C to elute the DNA.

22. The samples were centrifuged at 13,000 g for 3 min and the supernatant containing the modified DNA was transferred to a new tube.
23. The samples were stored at -20°C if required before proceeding to MSP.

## ***2.9 Real-Time Quantitative PCR (TaqMan)***

For TaqMan analysis cDNA was prepared from 2 µg of patient and control RNA that was first DNase I treated to eliminate contamination of genomic DNA. TaqMan probe based assay chemistry was used to determine gene expression levels of candidate genes in patient and control samples. TaqMan chemistry uses an oligonucleotide fluorescent probe to detect amplification of specific PCR products during the amplification reaction. The probe has a fluorescent reporter dye attached to the 5' end and a quencher dye on the 3' end. During PCR amplification the probe is cleaved by the 5' nuclease activity of the Taq DNA polymerase. This separates the reporter dye from the quencher dye to increase the signal of the reporter dye. With each cycle of PCR more reporter dye is released in proportion to the amount of PCR product generated. The TaqMan reactions were performed on the 5700 Applied Biosystems Sequence Detection System.

### ***2.9.1 DNase I Treatment of RNA***

The DNA-free reagent from Ambion (Austin, Texas, USA) was used to DNase treat all samples to remove contaminating DNA from RNA preparations. Additionally, divalent cations are removed which may otherwise degrade RNA when heated.

1. 2 µg of each RNA sample was added to a 1.5 ml microcentrifuge tube
2. 0.1 volume 10 x DNase I Buffer and 1 µl rDNase I were added to the RNA samples and mixed gently.

3. The samples were incubated at 37°C for 30 min.
4. The DNase Inactivation Reagent was resuspended and 0.1 volume or 2 µl, whichever was greater, was added to each sample and mixed well.
5. The samples were incubated at room temperature for 2 min with occasional mixing.
6. The samples were centrifuged at 10,000 g for 1.5 min and the RNA was transferred to a fresh microcentrifuge tube.

### ***2.9.2 First Strand cDNA Preparation***

Reverse transcription of cDNA from patient and control RNA templates was carried out using the RETROscript kit (Ambion).

1. For each sample 2 µg of DNase I treated total RNA was added to 2 µl of Random Decamers in a 1.5 ml microcentrifuge tube then nuclease free H<sub>2</sub>O was added to a final volume of 12 µl.
2. The samples were mixed, centrifuged briefly and heated at 75°C for 3 min to denature the RNA.
3. The tubes were placed on ice, centrifuged briefly and returned to ice.
4. The following RT components were then added to each tube:

10 x RT Buffer	2 µl
dNTP mix	4 µl
RNase Inhibitor	1 µl
MMLV Reverse Transcriptase	1 µl
5. The samples were mixed gently, centrifuged briefly and incubated at 42°C for 1 hr.

6. The samples were incubated at 95°C for 10 min to inactivate the Reverse Transcriptase and stored at -20°C until required.

### ***2.9.3 Real-Time Quantitative PCR (TaqMan) Analysis***

The 2 x TaqMan Universal PCR Master Mix (Applied Biosystems) was used for all 5' nuclease assays. For the majority of genes studied ready made TaqMan Gene Expression Assays (Applied Biosystems) were used. These contained primers and probe and were supplied as 20 x solutions. Where individual primer and probe sets were used primers and probes were diluted to 5 pmol/μl.

1. A PCR master mix was prepared for each assay for the required number of samples plus a negative control. The volumes of reagents for one sample using a ready made assay were as follows:

TaqMan Universal PCR Master Mix	12.5 μl
Gene Expression Assay	1.25 μl
Nuclease-free H <sub>2</sub> O	6.25 μl

The volumes of reagents for one sample using individual primers and probes were as follows:

TaqMan Universal PCR Master Mix	12.5 μl
Forward Primer	1.5 μl
Reverse Primer	1.5 μl
Probe	0.5 μl
Nuclease-free H <sub>2</sub> O	4 μl



2. 20 µl of the reaction mix was transferred to the required number of wells in a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems).
3. 5µl of cDNA (diluted 1:20) was added to the appropriate well of the plate and each sample was run in triplicate.
4. The plate was sealed with MicroAmp Optical caps (Applied Biosystems) and was centrifuged briefly.
5. The plate was transferred to the 5700 Sequence Detection System and PCR was carried out under the following conditions: Enzyme Activation at 95°C for 10 min followed by 45 cycles of 15 sec denaturation at 95°C and 1 min annealing/extension at 60°C.
6. The samples were analysed using the Sequence Detection System Software (Applied Biosystems).
7. To determine the relative gene expression ratio for each sample the  $2^{-\Delta\Delta C_t}$  method was used (Livak and Schmittgen 2001) which compares the average  $C_t$  of each sample to the average  $C_t$  of the normal controls to give a gene expression ratio.

## ***2.10 cDNA Microarray Experiments***

cDNA microarray slides were sourced from the Sanger Institute (Cambridge, UK). Each slide has 9,932 spotted cDNAs representing approximately 6,000 known genes and ESTs. Of these 8,817 cDNAs are derived from the IMAGE collection (HGMP), 647 cDNAs are derived from the IMAGE collection (Research Genetics) and 468 cDNAs from chromosome 22 gene-specific PCR products (Sanger Institute). The microarray slides are also spotted with heterologous cDNAs for five bacterial genes (*trp*, *lysA*, *thrB*, *dapB*, *pheB*) that are not present in mammalian RNA samples. Plasmids with inserts corresponding to each of the five bacterial genes were used as a template for in vitro

transcription and then mixed to produce a bacterial cocktail. The bacterial cocktail was added to each sample as a positive control and indicator of sensitivity on the array.

For each experiment total RNA extracted from one patient or control sample was compared with a pool of total RNA from normal control samples. The RNA samples were amplified and fluorescently labelled then hybridised together on the same microarray slide.

### ***2.10.1 Generation of a Bacterial mRNA Cocktail***

Plasmids with inserts corresponding to each of the five *Bacillus subtilis* cDNA clones were kindly provided by Jiangting Hu (Oxford). 5 µg of each plasmid was digested with the restriction endonuclease NotI (Promega) then in vitro transcribed using the T3 MEGAscript kit from Ambion.

1. A digestion mix was prepared for a total of 8 reactions:

Buffer D 10 x	16 µl
BSA 100 x	1.6 µl
NotI (80 U/µl)	1 µl

2. A digestion reaction was set up for each plasmid in a 0.5 ml microcentrifuge tube with the following components:

Plasmid DNA	volume for 5 µg
Digestion Mix	2.3 µl
H <sub>2</sub> O	to 20 µl

3. The tubes were incubated at 37°C for 2.5 hr.

4. The reactions were precipitated by addition of the following components:

0.5 M EDTA	1 $\mu$ l
3 M Sodium Acetate	2 $\mu$ l
100% Ethanol	40 $\mu$ l

5. The samples were vortexed and incubated at -20°C for 30 min.

6. The tubes were centrifuged at 13,000 rpm for 15 min in a microcentrifuge.

7. The supernatant was removed and the pellets were air-dried and resuspended in 10  $\mu$ l of nuclease-free H<sub>2</sub>O.

8. An in vitro transcription reaction was set up for each plasmid in a 0.5 ml microcentrifuge tube with the following reaction components:

Nuclease-free H <sub>2</sub> O	5 $\mu$ l
ATP solution (75 mM)	2 $\mu$ l
CTP solution (75 mM)	2 $\mu$ l
GTP solution (75 mM)	2 $\mu$ l
UTP solution (75 mM)	2 $\mu$ l
10 x Reaction Buffer	2 $\mu$ l
T3 Enzyme Mix	2 $\mu$ l
Linear plasmid DNA (~1 $\mu$ g)	3 $\mu$ l

9. The samples were incubated at 37°C for 5 hr.

10. The reactions were terminated by addition of 115  $\mu$ l of nuclease-free H<sub>2</sub>O and 15  $\mu$ l of ammonium acetate stop solution.

11. The samples were extracted with 150  $\mu$ l of phenol/chloroform and then with 150  $\mu$ l of chloroform.

12. Each aqueous phase was transferred to a new microcentrifuge tube.

13. 150  $\mu$ l of isopropanol was added to each tube to precipitate the RNA and samples were incubated for 30 min at -20°C.

14. The samples were centrifuged at 13,000 rpm for 15 min at 4°C.
15. The supernatant was removed and the RNA pellets were air-dried and resuspended in 20 µl of nuclease-free H<sub>2</sub>O.
16. The purified RNA samples were quantified using a spectrophotometer and visualised using agarose gel electrophoresis.
17. 3 volumes of 100% ethanol were added to the RNA samples.
18. 250 µl of a 10 x bacterial mRNA cocktail was prepared with the five mRNAs at the following concentrations:  
0.3 ng/µl *lysA*  
1.5 ng/µl *pheB*  
3 ng/µl *thrB*  
15 ng/µl *dapB*  
30 ng/µl *trp*
19. The cocktail was then diluted 10-fold to obtain a 1 x working solution.

### ***2.10.2 Generation of Fluorescently-Labelled aRNA targets***

The Amino Ally MessageAmp aRNA Kit (Ambion) was used to amplify input RNA from patient and normal control samples. This kit reliably amplifies starting material using a T7 based linear amplification method. It also allows for incorporation of the fluorescent Cy3 or Cy5 dyes.

#### ***2.10.2.1 cDNA Synthesis***

1. 2 µg of RNA from each sample to be amplified was placed into a 0.5 ml microcentrifuge tube.

2. 1 µl of T7 Oligo(dT) Primer and 2 µl of bacterial cocktail were added to the samples and the volume was made up to 12 µl with nuclease free H<sub>2</sub>O.

3. The samples were incubated for 10 min at 70°C then centrifuged briefly and placed on ice.

4. A reverse transcription master mix was assembled using the following components for one reaction:

10 x First Strand Buffer	2 µl
RNase Inhibitor	1 µl
dNTP Mix	4 µl

5. 7 µl of the reverse transcription master mix was added to each sample and the samples were placed in an incubator at 42°C.

6. 1 µl of reverse transcriptase was added to each sample in the incubator and the samples were incubated for 2 hr.

7. The tubes were placed on ice and the second strand cDNA synthesis components were added as follows:

cDNA from step 6	20 µl
Nuclease free H <sub>2</sub> O	63 µl
10 x Second Strand Buffer	10 µl
dNTP Mix	4 µl
DNA Polymerase	2 µl
RNase H	1 µl

8. The samples were mixed gently and centrifuged briefly before incubating at 16°C for 2 hr.

9. For each sample one cDNA Filter cartridge was equilibrated by adding 50 µl of cDNA binding buffer and incubating for 5 min.

10. 250 µl of cDNA Binding Buffer was added to each sample and vortexed then the samples were added to the cDNA filter cartridges in 2 ml collection tubes.
11. The samples were centrifuged at 10,000 g for 1 min and the flow through was discarded.
12. 650 µl of cDNA wash Buffer was added to each cDNA Filter Cartridge and centrifuged for 1 min at 10,000 g.
13. The flow through was discarded and the cDNA Filter Cartridges were centrifuged for 1 min to remove any residual ethanol.
14. Each cDNA Filter Cartridge was transferred to a fresh 1.5 ml tube.
15. 9 µl of nuclease free H<sub>2</sub>O heated to 50°C was added to each filter and incubated for 2 min. The tubes were then centrifuged for 1.5 min at 10,000 g.
16. Step 15 was repeated and the cDNA filter cartridges were discarded.
17. If required the volume of each cDNA sample was increased to 14 µl with nuclease free H<sub>2</sub>O and stored at -20°C until required.

#### ***2.10.2.2 In Vitro Transcription to Synthesise aRNA***

1. The *in vitro* transcription reaction components were assembled at room temperature. The volume of reagents for one sample was as follows:

double stranded cDNA	14 µl
aaUTP Solution	3 µl
ATP, CTP, GTP Mix	12 µl
UTP Solution	3 µl
T7 10 x Reaction Buffer	4 µl
T7 Enzyme Mix	4 µl

2. The reactions were incubated at 37°C for 14 hr in an air incubator.

3. 2 µl of DNase I was added to each sample and mixed gently then centrifuged briefly.
4. The samples were incubated at 37°C for 30 min.
5. One aRNA Filter Cartridge for each sample was equilibrated by adding 100 µl of aRNA binding buffer and incubating for 5 min.
6. Nuclease free H<sub>2</sub>O was added to each sample to bring the total volume to 100 µl and the samples were mixed gently.
7. 350 µl of aRNA Binding Buffer was added to each sample and mixed gently.
8. 250 µl of 100% Ethanol was added to each sample and mixed gently.
9. The samples were added to the equilibrated aRNA Filter Cartridges in 2 ml collection tubes and centrifuged for 1 min at 10,000 g and the flow through was discarded.
10. 650 µl of aRNA Wash Buffer was added to each filter and centrifuged for 1 min at 10,000 g and the flow through was discarded.
11. Each aRNA Filter Cartridge was transferred to a fresh 1.5 ml tube.
12. 50 µl of nuclease free H<sub>2</sub>O heated to 50°C was added to each filter and incubated for 2 min. The tubes were then centrifuged for 1.5 min at 10,000 g.
13. Step 12 was repeated and the cDNA filter cartridges were discarded.

#### ***2.10.2.3 aRNA Dye Coupling and Clean up***

1. The concentration of each aRNA sample was determined by spectrophotometer.
2. 15 µg of each sample was transferred to a fresh tube and vacuum dried to 7 µl.
3. 9 µl of Coupling Buffer was added to each sample and mixed.
4. 4 µl of FluoroLink Cy3 (normal pool) or Cy5 (individual sample) NHS ester dye (GE Healthcare, Bucks, UK) was added to each sample and mixed.

5. The samples were incubated for 30 min at room temperature in the dark.
6. 4.5 µl of 4 M Hydroxylamine was added to each sample to quench the reaction and incubated for 15 min at room temperature in the dark.
7. The dye labelled aRNA samples were purified as described in section 2.10.2.2 starting at step 5.

### ***2.10.3 Competitive Hybridisation of Labelled Targets onto Microarrays***

1. The two labelled targets were combined as follows:

Labelled target 1 (Cy3-labelled)	33 µl
Labelled target 2 (Cy5-labelled)	33 µl
PolyA DNA (2 µg/µl)	3 µl
Human Cot1 DNA (2 µg/µl)	3 µl
3 M Sodium Acetate pH 5.2	7 µl
100% Ethanol	237 µl

2. The nucleic acids were precipitated at -70°C for 30 min.
3. The samples were centrifuged at 13,000 rpm for 10 min to pellet the precipitate.
4. The pellets were washed in 200 µl of 70% ethanol and centrifuged for 1 min then air dried.
5. The pellets were resuspended in 30 µl of hybridisation buffer (5X SSC, 6X Denhardt's solution, 60 mM Tris HCl pH 7.6, 0.12% sarkosyl, 48% formamide) and 6 µl of nuclease free H<sub>2</sub>O.
6. The hybridisation mixture incubated in a dry block at 100°C for 5 min then cooled for 10 min at room temperature and centrifuged briefly.



7. For each hybridisation one coverslip and one microarray slide were cooled to 4°C in a cold room to reduce non specific binding of the labelled targets to the surface of the slide.
8. In the cold room, the hybridisation mixture was applied to the centre of the coverslip and the microarray slide was inverted and lowered onto the coverslip to allow the hybridisation mixture to spread evenly across the microarray.
9. The microarray slide was placed in a sealed humid chamber to prevent buffer evaporation and incubated at 47°C overnight in the dark.
10. The microarrays were removed from the humid chambers and placed 2 x SSC until the coverslip slid off.
11. The microarrays were washed with gentle shaking in 2 x SSC for 5 min at room temperature.
12. The microarrays were transferred to 0.1 x SSC, 0.1% SDS and washed for 30 min at room temperature.
13. Step 12 was repeated.
14. The microarrays were transferred to 0.1 x SSC and washed for 5 min at room temperature.
15. Step 14 was repeated.
16. The microarrays were centrifuged at 1,000 rpm for 1 min to dry the slides.

#### ***2.10.4 Scanning and Image Quantitation***

The microarray slides were scanned using the ScanArray 4000 microarray scanner (PerkinElmer, Boston, MA) with a 10 µm resolution. This generated one image for the Cy3 channel scanning at wavelength 532 nm, and one image for the Cy5 channel scanning at wavelength 635 nm. The images were saved as TIFF files and imported into

the image quantitation software QuantArray 3.0 (PerkinElmer). Using QuantArray the Cy-3 image was given a red colour and the Cy5 image was given a green colour then the two images were overlaid. The top left spot of the array was located manually to allow the software to display a grid of circles organised to locate each spot on the array. If required, the grid was adjusted manually to accurately match the location of the spots on the array. The QuantArray software then calculated the signal intensity for each spot in the Cy3 and Cy5 channels. This was calculated from the 60th-95th percentile interval to reduce background signal by the exclusion of low intensity pixels and give lower weight to saturated pixels. The intensities of the Cy3 and Cy5 signals were then normalised to the median value of the array as a whole and the ratio for each spot was obtained. The data was exported to the GeneSpring software as a tab delimited text file for data analysis.

#### ***2.10.5 Data Analysis***

Tab delimited text files were imported into the GeneSpring software package (Silicon Genetics, Redwood City, CA) and data analysis was performed. The data were firstly log transformed to give an approximately normal distribution. Lists of genes with increased or decreased expression were generated by filtering by expression level. Typically an expression ratio cut off of  $>0.2$  was selected for genes with increased expression and a cut off of  $<0.5$  was selected for genes with decreased expression. In order to compare groups of samples the t-test or ANOVA based on a parametric Welch's t-test without assumption of equality of variances was used. The false discovery rate was controlled by either the Benjamini-Hochberg multiple testing correction or the more conservative Bonferroni multiple testing correction. Hierarchical clustering was performed using standard correlation.

## 2.11 Affymetrix GeneChip Experiments

The Affymetrix Human GeneChip U133 Plus 2.0 whole genome expression array (Affymetrix, Santa Clara, CA) was used for this study. The probe sets on these arrays were identified from the GenBank dbEST and RefSeq databases and the sequence clusters were created from the UniGene database build 133 (April 2001). These sequence clusters were then refined by analysis and comparison with public databases such as the University of California Santa Cruz Golden-Path human database (April 2001). There are an additional 9,921 probe sets representing a further 6,500 genes from the GenBank dbEST and RefSeq databases and the sequence clusters were created from the UniGene database build 159 (January 2003). These sequence clusters were then refined by analysis and comparison with public databases such as NCBI human genome assembly (Build 31). This results in an array that can provide an analysis of over 47,000 transcripts representing 39,000 human genes. The complimentary oligonucleotide probes are synthesised directly on the array. These comprise of eleven pairs of oligonucleotide probes corresponding to each sequence represented on the array. Total RNA samples from patients or normal controls were amplified and biotin labelled using the Two-Cycle Target Labelling and Control Reagent kit (Affymetrix) and one sample was hybridised to one array. Poly-A controls were added to the starting RNA as internal controls for amplification, labelling, assay sensitivity, and data consistency. These controls hybridise to the internal control probe sets for the *B. subtilis* genes *lys*, *phe*, *thr*, and *dap* that are contained on the array. The arrays were washed and stained before being scanned with an Affymetrix Gene Chip Scanner 3000.

### ***2.11.1 Target Amplification and Labelling***

#### ***2.11.1.1 cDNA Synthesis, Cycle 1***

1. A mix of the T7-oligo(dT) primer and poly-A controls was prepared for ten reactions as detailed below:

T7-oligo(dT) primer (50 $\mu$ M)	2 $\mu$ l
Diluted poly-A controls	2 $\mu$ l
RNase-free H <sub>2</sub> O	16 $\mu$ l

2. Each total RNA sample was mixed with the T7-oligo(dT) primer/poly-A controls mix in the following quantities:

Total RNA	50 ng
T7-oligo(dT) primer/poly-A controls mix	2 $\mu$ l
RNase-free H <sub>2</sub> O	to 5 $\mu$ l

3. The samples were incubated at 70°C for 6 min then snap-chilled on ice for 2 min.

4. The following First-strand Master-Mix components were added to each sample:

5 x 1st Strand Reaction Mix	2 $\mu$ l
0.1 M DTT	1 $\mu$ l
RNase Inhibitor	0.5 $\mu$ l
10 mM dNTPs	0.5 $\mu$ l
SuperScript II	1 $\mu$ l

5. The samples were incubated at 42°C for 1 hr.

6. The samples were heated at 70°C for 10 min to inactivate the RT enzyme then cooled at 4°C for 2 min.

7. The following Second-strand Master-Mix components were added to each sample

RNase-free H <sub>2</sub> O	4.8 µl
17.5 mM MgCl <sub>2</sub> ,	4.0 µl
10 mM dNTPs	0.4 µl
<i>E. coli</i> DNA Polymerase I	0.6 µl
RNase H	0.2 µl

8. The samples were incubated at 16°C for 2 hr.

9. The samples were heated at 70°C for 10 min then cooled at 4°C for 2 min.

#### ***2.11.1.2 In Vitro Transcription, Cycle 1***

The MEGAScript T7 kit (Ambion) was used for in vitro transcription (IVT) amplification of the samples.

1. The following IVT components were added to each of the double-stranded cDNA samples:

10 x Reaction Buffer	5 µl
ATP Solution	5 µl
CTP Solution	5 µl
UTP Solution	5 µl
GTP Solution	5 µl
Enzyme Mix	5 µl

2. The samples were incubated at 37°C for 16 hr in an air incubator.

### *2.11.1.3 cRNA Clean Up, Cycle 1*

1. 50 µl of RNase-free H<sub>2</sub>O was added to each IVT reaction and the samples were mixed by vortexing.
2. 350 µl of IVT cRNA Binding Buffer was added to each sample and the tubes were mixed by vortexing.
3. 250 µl of 100% ethanol was added to the samples and the tubes were mixed well by pipetting.
4. Each sample was applied to an IVT cRNA Cleanup Spin Column in a 2 ml collection tube and centrifuged for 15 sec at 12,000 rpm. The flow-through and collection tube were discarded and the spin columns were transferred to new 2 ml collection tubes.
5. 500 µl of IVT cRNA Wash Buffer was added to the spin columns. The columns were centrifuged for 15 sec at 12,000 rpm and the flow-through was discarded.
6. 500 µl of 80% ethanol was added to the spin columns. The columns were centrifuged for 15 sec at 12,000 rpm and the flow-through was discarded.
7. The columns were centrifuged for 5 min at 13,000 rpm with the caps open to dry the membrane. The flow-through and collection tube were discarded and the spin columns were transferred to new 1.5 ml collection tubes.
8. 13 µl of RNase-free H<sub>2</sub>O was pipetted onto the membrane of each spin column and the columns were centrifuged for 1 min at 13,000 rpm to elute the cRNA.
9. cRNA yield was determined by spectrophotometry and 600 ng of cRNA was used for the second round of amplification.

#### 2.11.1.4 cDNA Synthesis, Cycle 2

1. 2  $\mu$ l of diluted random primers (0.2  $\mu$ g/ $\mu$ l) were added to 600 ng of cRNA and H<sub>2</sub>O was added to a final volume of 11  $\mu$ l.

2. The samples were incubated at 70°C for 10 min then snap-chilled on ice for 2 min.

3. The following First-strand Master-Mix components were added to each sample

5 x 1st Strand Reaction Mix	4 $\mu$ l
0.1 M DTT	2 $\mu$ l
RNase Inhibitor	1 $\mu$ l
10 mM dNTPs	1 $\mu$ l
SuperScript II	1 $\mu$ l

4. The samples were incubated at 42°C for 1 hr then cooled at 4°C for 2 min.

5. 1  $\mu$ l of RNase H was added to each sample and the tubes were incubated at 37°C for 20 min.

6. The samples were heated at 95°C for 5 min then cooled at 4°C for 2 min.

7. 4  $\mu$ l of diluted T7-oligo(dT) primer (5  $\mu$ M) was added to each sample and the tubes were incubated at 70°C for 6 min then cooled at 4°C for 2 min.

8. The following Second-strand Master-Mix components were added to each sample:

RNase-free H <sub>2</sub> O	88 $\mu$ l
5 x 2nd Strand Reaction Mix	30 $\mu$ l
10 mM dNTPs	3 $\mu$ l
<i>E.coli</i> DNA Polymerase I	4 $\mu$ l

9. The samples were incubated at 16°C for 2 hr.

10. 2 µl of T4 DNA Polymerase was added to each sample and the tubes were incubated at 16°C for 10 min then cooled at 4°C for 2 min.

#### ***2.11.1.5 Double-Stranded cDNA Cleanup, Cycle 2***

1. 600 µl of cDNA Binding Buffer was added to each sample and mixed by vortexing.
2. 500 µl of each sample was added to a cDNA Cleanup Spin Column in a 2 ml collection tube. The columns were centrifuged for 1 min at 12,000 rpm and the flow-through was discarded.
3. The remainder of the sample was added to the spin column and centrifuged for 1 min at 12,000 rpm. The flow-through and collection tube were discarded and the spin columns were transferred to new 2 ml collection tubes.
4. 750 µl of cDNA Wash Buffer was added to the spin columns. The columns were centrifuged for 1 min at 12,000 rpm and the flow-through was discarded.
5. The columns were centrifuged for 5 min at 13,000 rpm with the caps open to dry the membrane. The flow-through and collection tube were discarded and the spin columns were transferred to new 1.5 ml collection tubes.
6. 14 µl of cDNA Elution Buffer was pipetted onto the membrane of each spin column and the columns were incubated for 1 min at room temperature then centrifuged for 1 min at 13,000 rpm to elute the cDNA.

#### ***2.11.1.6 Synthesis of Biotin-labelled cRNA***

1. The following IVT components were added to 12 µl of double-stranded cDNA:

RNase-free H<sub>2</sub>O

8 µl



10 x IVT Labelling Buffer	4 µl
IVT Labelling NTP Mix	12 µl
IVT Labelling Enzyme Mix	4 µl

2. The samples were incubated at 37°C for 16 hr in an air incubator.

#### ***2.11.1.7 Cleanup and Quantification of Biotin-Labelled cRNA***

1. 60 µl of RNase-free H<sub>2</sub>O was added to each IVT reaction and the samples were mixed by vortexing.
2. 350 µl of IVT cRNA Binding Buffer was added and the samples were mixed by vortexing.
3. 250 µl of 100% ethanol was added to the samples and mixed well by pipetting.
4. Each sample was added to an IVT cRNA Cleanup Spin Column in a 2 ml collection tube and centrifuged for 15 sec at 12,000 rpm. The flow-through and collection tube were discarded and the spin columns were transferred to new 2 ml collection tubes.
5. 500 µl of IVT cRNA Wash Buffer was added to the spin columns. The columns were centrifuged for 15 sec at 12,000 rpm and the flow-through was discarded.
6. 500 µl of 80% ethanol was added to the spin columns. The columns were centrifuged for 15 sec at 12,000 rpm and the flow-through was discarded.
7. The columns were centrifuged for 5 min at 13,000 rpm with the caps open to dry the membrane. The flow-through and collection tube were discarded and the spin columns were transferred to new 1.5 ml collection tubes.
8. 11 µl of RNase-free H<sub>2</sub>O was pipetted onto the membrane of each spin column and the columns were centrifuged for 1 min at 13,000 rpm to elute the cRNA.

9. 10 µl of RNase-free H<sub>2</sub>O was pipetted onto the membrane of each spin column and the columns were centrifuged for 1 min at 13,000 rpm to elute the cRNA.
10. cRNA yield was determined by spectrophotometry.

### ***2.11.2 Fragmentation of Biotin-Labelled cRNA***

The Fragmentation Buffer used for this step allows the break down of full length cRNA to 35-200 base fragments by metal induced hydrolysis.

1. The following components were added to 20 µg of biotin-labelled cRNA:
 

5 x Fragmentation Buffer	8 µl
RNase-free H <sub>2</sub> O	to 40 µl
2. The samples were incubated at 94°C for 35 minutes and then cooled on ice.

### ***2.11.3 Target Hybridisation***

1. Each sample was mixed in a hybridisation cocktail as follows:

Fragmented cRNA (0.5 µg/µl)	30 µl (15 µg)
Control Oligonucleotide B2 (3 nM)	5 µl
20 x Eukaryotic Hybridisation Controls (bioB, bioC, bioD, cre)	15 µl
Herring Sperm DNA (10 mg/ml)	3 µl
Bovine Serum Albumin (50 mg/ml)	3 µl
2 x Hybridisation Mix	150 µl
DMSO	30 µl
H <sub>2</sub> O	64 µl

2. The hybridisation cocktail was incubated at 99°C for 5 min and then at 45°C for 5 min.
3. The samples were centrifuged at 13,000 rpm for 5 min to remove any insoluble material from the hybridisation mixture.
4. The Affymetrix arrays were equilibrated to room temperature to prevent cracking of the rubber septa.
5. Each array was filled through one of the septa with 200 µl of 1 x Hybridisation Buffer.
3. The arrays were incubated at 45°C for 10 min with rotation at 60 rpm in an Affymetrix Hybridisation Oven.
4. The 1 x Hybridisation Buffer was removed and the array cartridge was filled with 200 µl of the hybridisation cocktail.
5. The arrays were incubated in the hybridisation oven at 45°C for 16 hr with rotation at 60 rpm.

#### ***2.11.4 Washing and Staining***

The washing and staining protocol uses the following solutions:

Wash Buffer A: Non-Stringent Wash Buffer (6 x SSPE, 0.01% Tween-20)

Wash Buffer B: Stringent Wash Buffer (100 mM MES, 0.1 M [Na<sup>+</sup>], 0.01% Tween-20)

Stain Buffer (100 mM MES, 1 M [Na<sup>+</sup>], 0.05% Tween-20)

SAPE Solution Mix (1 x Stain Buffer, 2 mg/ml BSA, 10 µg/ml Streptavidin Phycoerythrin-SAPE)

Antibody Solution Mix (1 x Stain Buffer, 2 mg/ml BSA, 0.1 mg/ml Goat IgG, 3 µg/ml Anti-streptavidin biotinylated antibody)

1. After the 16 hr hybridisation, the hybridisation cocktail was removed from the array and 250 µl of Wash Buffer A was added to completely fill the array.

2. The arrays were washed and stained in the Affymetrix Fluidics Station 450, using the EukGE-WS2v5 fluidics script as follows:

Post Hyb Wash #1 10 cycles of 2 mixes/cycle with Wash Buffer A at 30°C

Post Hyb Wash #2 6 cycles of 15 mixes/cycle with Wash Buffer B at 50°C

1<sup>st</sup> Stain Stain the array for 5 min in SAPE solution at 35°C

Post Stain Wash 10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C

2nd Stain Stain the array for 5 min in antibody solution at 35°C

3rd Stain Stain the array for 5 min in SAPE solution at 35°C

Final Wash 15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C.

The holding temperature was 25°C.

#### ***2.11.5 Scanning and experiment quality control***

The Affymetrix chips were immediately scanned using the Affymetrix GeneChip Scanner 3000 with a 3 µm pixel resolution at 570 nm wavelength. When the scan was completed the GCOS automatically aligned a grid on the image for probe cell identification and calculated the probe cell intensity data using the Cell Analysis algorithm. All chips were scaled to a target intensity of 100 to control image brightness and to render all chips comparable. To achieve this the intensity values were multiplied by a scaling factor to reach the target level of 100. The scaling factor should be comparable for all samples and less than 3. The Average Background values were typically around 50-70, and the acceptable range is 20-100. The number of probe sets identified as “present” by the GCOS was expressed as a percentage of the total number of probe sets on the array and was approximately 40% for all samples. The

Hybridisation Controls added to the hybridisation cocktail were identified as “present” by the GCOS. The signal values of the 3’ and 5’ probe sets for the GAPDH gene were used to assess RNA integrity and amplification. The ratio of the 3’ to the 5’ probe set was calculated for all samples and although the expected ratio would be 1 the acceptable level is a value less than 3. The amplification method used in these experiments has a 3’ bias due to the T7 promoter resulting in transcription of cRNA from the sense strand of the cDNA. The data was exported to the GeneSpring software as a tab delimited text file or .CEL file for data analysis.

***Chapter 3***

***Gene expression analysis of***

***telomerase related genes in Chronic***

***Myeloid Leukaemia***

**Associated Publication:** *hTERT*, the catalytic component of telomerase, is downregulated in the haematopoietic stem cells of patients with chronic myeloid leukaemia.

L J Campbell, C Fidler, H Eagleton, A Peniket, R Kusec, S Gal, T J Littlewood, J S Wainscoat and J Boulton. *Leukemia* (2006) 20, 671–679

### **3.1 Introduction**

#### **3.1.1 Chronic Myeloid Leukaemia**

Chronic Myeloid Leukaemia (CML) is a clonal myeloproliferative disorder that originates in a pluripotent haematopoietic stem cell (HSC) (Shet *et al.* 2002). The disease is characterised by an excessive proliferation of granulocytes (Hehlmann *et al.* 2007). Disease incidence is rare with 1 or 2 cases per 100,000 head of population per year and median age of onset is 65 years (Hehlmann *et al.* 2007). CML is consistently associated with an acquired genetic abnormality: the Philadelphia (Ph) chromosome (Nowell and Hungerford 1960; Rowley 1973). This is a rearrangement between the major breakpoint cluster region (BCR) on chromosome 22 and the c-ABL proto-oncogene on chromosome 9 (Shet *et al.* 2002). This results in a t(9;22) translocation encoding the BCR/ABL oncogenic fusion gene. This gene encodes the p210 BCR/ABL, an oncogenic fusion protein with increased tyrosine kinase activity that can induce cytoskeletal abnormalities which may increase cell motility (Salgia *et al.* 1997). The fusion protein enhances cell survival by conferring resistance to apoptosis (Bedi *et al.* 1994). The translocation is the hallmark of CML and is found in up to 95% of patients (Groffen *et al.* 1984). The translocation has been demonstrated to be sufficient and necessary for malignant transformation (Daley *et al.* 1990; Lugo *et al.* 1990). Transplantation of BCR/ABL transduced cells into animal models induces CML like syndromes and the mouse model is a very accurate model of the human disease (Van Etten 2002).

In patients the disease inevitably progresses through a series of stages beginning with a chronic phase (CP) lasting 3-4 years where myeloid progenitors and mature cells

accumulate in the blood (Shet *et al.* 2002). After this time the disease can transform to accelerated phase (AP) and terminates in blast crisis (BC), in which transformation to an acute leukaemia with myeloid, B-lymphoid, or occasionally T-lymphoid features occurs (Martin *et al.* 1980; Allouche *et al.* 1985; Kantarjian *et al.* 1987; Calabretta and Perrotti 2004). The Ph chromosome is typically the only cytogenetic abnormality reported in CP patients, but cells with additional chromosomal abnormalities are commonly present at BC. Approximately 80% of patients show additional chromosome changes in BC, the most common of which are extra copies of the Philadelphia chromosome, trisomy 8, isochromosome 17, trisomy 19 and complex karyotypes (Hehlmann and Saussele 2008). Mutations are also relatively common with 25% of myeloid BC patients having p53 mutation and 50% of lymphoid BC having p16/AKT mutation (Ahuja *et al.* 1989; Hehlmann and Saussele 2008). Additionally, expression of the BCR/ABL fusion protein results in an unstable genome in the haematopoietic stem cell. Increasing levels of BCR/ABL lead to inhibition of DNA repair allowing the accumulation of genetic defects and disease progression (Kharbanda *et al.* 1997; Deutsch *et al.* 2001).

For many years the only curative treatment of CML was an allogeneic bone marrow transplant, however many patients were excluded from this due to age or donor availability (Mughal and Goldman 2004). Treatment of patients with CML was revolutionised with the first clinical use of imatinib mesylate (Gleevec; STI571) (Druker *et al.* 2001; Druker *et al.* 2001). This is a highly specific tyrosine kinase inhibitor of the BCR/ABL fusion protein (Druker *et al.* 1996). Imatinib blocks the kinase activity of BCR/ABL by competing with ATP for the enzymatic binding site and significantly reduces the number of leukaemic cells (Druker *et al.* 1996). Many newly diagnosed patients are now being successfully treated with this drug (Mughal and Goldman 2004).



### 3.1.2 Telomeres and Telomerase in CML

A reduction in telomere length has been demonstrated to associate with disease evolution in CML. It has been demonstrated that telomere length in AP and BC is reduced compared with serial samples taken at CP in patients with CML (Boultonwood *et al.* 1999). Furthermore telomere shortening observed at the time of diagnosis in CML significantly influences the time of progression to AP suggesting that the measurement of diagnostic telomere length may be clinically important in the selection of patients at high risk of disease transformation in CML (Boultonwood *et al.* 2000). Similarly, Brummendorf and colleagues have shown that patients with CML, in whom the blast phase subsequently developed within 2 years, had significantly shorter telomeres than those in whom the blast phase did not develop for at least 2 years (Brummendorf *et al.* 2000). Telomerase activity has been investigated in the bone marrow cells of patients with CML during disease progression (Tatematsu *et al.* 1996; Ohyashiki *et al.* 1997; Verstovsek *et al.* 2003; Bock *et al.* 2004). These studies measured telomerase activity rather than expression of *hTERT* in peripheral blood or bone marrow mononuclear cells of CML patients and reported an increase in telomerase activity. It was reported that telomerase activity increased with disease progression of CML. An increase in telomerase activity has been correlated with a decrease in telomere length in these studies. This apparent paradox has been explained by the suggestion that a critical point of telomere length is reached and only those cells with increased telomerase activity can survive (Ohyashiki *et al.* 1997).

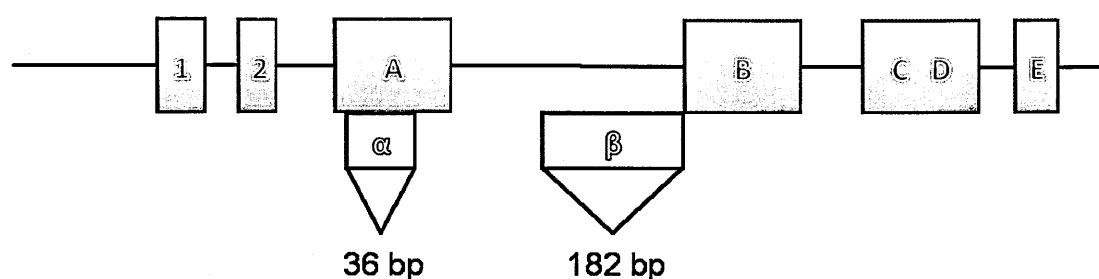
### **3.1.3 *hTERT* expression as a marker for telomerase activity**

There is a strong correlation between the presence of *hTERT* mRNA and telomerase activity and it is recognized that *hTERT* is the rate-limiting determinant of the enzymatic activity of human telomerase (Weinrich *et al.* 1997; Counter *et al.* 1998; Nakayama *et al.* 1998). Although various steps at post-transcriptional and post-translational level can modulate *hTERT* function the transcriptional control of the gene is a major contributor to the regulation of telomerase activity in human cells (Poole *et al.* 2001; Aisner *et al.* 2002). Nakayama *et al.* demonstrated that ectopic *hTERT* expression is sufficient to confer enzymatic activity to telomerase negative cells and showed that *hTERT* expression is the primary determinant regulating telomerase activity (Nakayama *et al.* 1998). It is widely accepted that *hTERT* expression levels may be used as a surrogate marker for telomerase activity (Dome *et al.* 1999; Bieche *et al.* 2000; de Kok *et al.* 2000). Indeed, several groups consider that the measurement of *hTERT* levels by quantitative RT-PCR is a more accurate and sensitive means of quantitation and detection than the TRAP assay (Dome *et al.* 1999; Bieche *et al.* 2000; de Kok *et al.* 2000).

### **3.1.4 Telomerase splice variants**

Posttranscriptional mechanisms play a role in the regulation of telomerase. Several splice variants of the *hTERT* gene have been identified (Fig 3.1) that have different activity as a result of truncations in domains with catalytic activity (Ulaner *et al.* 1998). The full length transcript is known as  $+\alpha+\beta$  and is fully functional (Yi *et al.* 2000). The  $-\alpha+\beta$  transcript has a 36bp deletion causing an in frame deletion of the reverse transcriptase motif that is a dominant negative inhibitor of *hTERT* enzyme activity

(Colgin *et al.* 2000). The + $\alpha$ - $\beta$  is a 182bp deletion that causes a nonsense mutation before the RT domain causing a non-functional protein to be formed (Ulaner *et al.* 1998). When both of the above sites are deleted this results in a - $\alpha$ - $\beta$  transcript which is an inactive truncated protein (Yi *et al.* 2000). Differences in expression of these splice variants can be studied by measuring RT-PCR products on the Agilent Bioanalyser to reveal the size and quantity of each band (Keith and Hoare 2004).



**Figure 3.1 Telomerase splice variants.** Representation of the coding region of human telomerase reverse transcriptase. The reverse transcriptase domains are shown as filled boxes and labelled 1, 2 and A-E (Nakamura *et al.* 1997). Locations of the 36bp  $\alpha$  deletion and the 182 bp  $\beta$  deletion are shown as open boxes (Ulaner *et al.* 2000).

### 3.1.5 Regulation of Telomerase

The proto-oncogene *c-Myc*, a positive regulator of telomerase, is one of the major determinants of *hTERT* expression. It acts by directly binding the *hTERT* promoter to induce transcription. Kyo *et al.* have shown that expression of *c-Myc* and *SP1* correlates with transcription of *hTERT* and suggest that *c-Myc* and *SP1* cooperatively function as the main regulators of *hTERT* expression (Kyo *et al.* 2000). *c-Myc* binds a site known as the E-box (CACGTG) and *SP1* binds a GC-box sequence (Takakura *et al.* 1999). *c-MYC* and *hTERT* expression are found to be increased in parallel in several malignancies such as prostate cancer, cervical cancer and lipomatous tumours and it is

likely that increased *c-MYC* expression leads to increased *hTERT* expression in these diseases (Latil *et al.* 2000; Sagawa *et al.* 2001; Schneider-Stock *et al.* 2003).

A potent inhibitor of telomerase protein activity, the TRF1–interacting protein PinX1, has been identified. Telomerase activity is inhibited by direct binding of PinX1 to the catalytic subunit of telomerase, so that the RNA template cannot bind (Zhou and Lu 2001; Banik and Counter 2004). Increased PinX1 expression leads to reduced telomerase activity and shortened telomeres (Zhou and Lu 2001). Recently it has been demonstrated that PinX1 directly interacts with TRF1 and this interaction allows TRF1 to recruit PinX1 to telomeres which is required for PinX1 to prevent telomere elongation (Soohoo *et al.* 2011).

### **3.1.6 Telomerase holoenzyme**

Human telomerase is a ribonucleoprotein of which the minimum components are human telomerase RNA component (hTR), human telomerase catalytic subunit (hTERT) and TEP1, a telomerase associated protein. TEP1 is not the rate limiting factor for telomerase activity but it is involved in enzyme activity and is thought to be required for full activity of the enzyme (Chang *et al.* 2002). *hTR*, is commonly expressed in both malignant and normal cells and several studies have shown that *hTR* is up-regulated during tumorigenesis (Avilion *et al.* 1996; Blasco *et al.* 1996). Expression of *hTR* has previously been determined in many cancers and it generally does not correlate with *hTERT* expression levels. Unlike *hTERT*, the expression of *hTR* does not predict the presence or amount of telomerase activity (Avilion *et al.* 1996; Blasco *et al.* 1996).

### **3.1.7 Telomeric associated proteins**

Over recent years a number of telomeric-associated proteins have been identified. The interaction between the telomeric-associated proteins telomeric-repeat binding factor protein-1 (TRF1), Tin2 and tankyrase is organized to control the access of telomerase to TTAGGG telomeric repeats. A Tin2/TRF1 complex binds telomeric DNA and inhibits the access of telomerase to the telomeres to prevent the addition of more repeats (Smogorzewska and de Lange 2004). Tankyrase is a TRF1-interacting factor and tankyrase-mediated ADP-ribosylation of TRF1 leads to the release of TRF1 from telomeres, opening up the telomeric complex and permitting access to telomerase (Smith *et al.* 1998; Smith and de Lange 2000; Cook *et al.* 2002). Since Tankyrase inhibits binding of TRF1 to telomeric repeats *in vitro* it has been suggested that tankyrase regulates TRF1 and therefore might control telomere dynamics *in vivo* (Smith and de Lange 2000). Telomeric-repeat binding factor protein-2 (TRF2) mainly functions in telomere end-protection and, by restricting telomere elongation, is a negative regulator of telomere length (van Steensel *et al.* 1998). Thus telomerase and tankyrase are positive regulators of telomere length, whilst TRF1, TRF2 and PinX1 are negative regulators. Telomere length in normal and malignant cells is regulated by a delicate balance between these factors.

### **3.1.8 Aims of the study**

The aim of this study was to investigate the expression levels of the components of telomerase, its regulators and several telomeric-associated proteins in the HSCs of CML patients during disease progression in order to gain insight into the biology of telomerase and its regulation in CML during disease evolution.

## ***3.2 Materials and Methods***

### ***3.2.1 Samples***

Patient samples were sourced from either patients attending the John Radcliffe Hospital or from Dr. Rajko Kusek (University Hospital Merkur, Croatia). Bone marrow aspirates were obtained from patients with CML and in some cases peripheral blood was obtained and CD34<sup>+</sup> cells were then isolated. The clinical details of these patients are detailed in table 3.1. Normal control CD34<sup>+</sup> cells were obtained from bone marrow samples from patients attending the John Radcliffe Hospital for hip replacement surgery.

**Table 3.1 Clinical Details of CML patients.**

M=Male, F=Female, STI= treatment with Imatinib, INF= treatment with interferon alpha, CP=Chronic Phase, AP=Accelerated Phase, BC=Blast Crisis.

Identifier	Sex	Age (yrs)	Treatment at time of sampling	Sampling time (post diagnosis)	Remission status	Cytogenetics
Pt 1	F	66	INF	12 yrs	CP	46,XX,t(9;22)(q34;q11)
Pt 13	F	36	Hydroxyurea	7 yrs	CP	46,XX,t(1;2)(q11;p11),t(9;22)(q34;q11),inv(10)(p13q22)
Pt 14	F	42	none	at diagnosis	CP	46,XX,t(9;22)(q34;q11)
Pt 15	M	64	none	at diagnosis	CP	46,XY,t(9;22)(q34;q11)
Pt 19	M	26	INF	1 mth	CP	46,XY,t(9;22)(q34;q11)
Pt 26	M	61	none	at diagnosis	CP	46,XY,?(9;22;19)[7]/48,idem,+8,+der(22)t(9;22)[3]
Pt 28	M	34	none	at diagnosis	CP	46,XY,t(9;22;14)
Pt 29	F	27	none	at diagnosis	CP	46,XX,t(9;22)(q34;q11)
Pt 31	M	58	none	at diagnosis	CP	46,XY,t(9;22)(q34;q11)
Pt 20	F	68	Hydroxyurea and INF	4 yrs	AP	46,XX,t(9;22)[4]/46,idem,I(17)[6]
Pt 27	M	79	Hydroxyurea	4 yrs	AP	46,XY,t(9;22)(q34;q11)
Pt 18	M	33	Hydroxyurea	11 yrs	BC	46,XY,t(9;22)(q34;q11)
Pt 32	M	51	Hydroxyurea	8 yrs	BC	46,XY,t(9;22)(q34;q11)
Pt 33	F	18	Hydroxyurea	2 yrs	BC	46,XX,t(9;22)(q34;q11)
Pt 34	F	77	Hydroxyurea	9 yrs	BC	46,XX,t(9;22)(q34;q11)
Pt 35	M	49	Hydroxyurea	2 yrs	BC	46,XY,t(9;22)(q34;q11)
Pt 36	M	65	Hydroxyurea and INF	11 yrs	BC	46,XY,t(9;22)(q34;q11)
Pt 3	M	58	STI	3 mth	STI in remission	46,XY,der(9),t(9;22)?del(22)(q13),der(22)t(9;22)(q34;q11) 0/50
Pt 4	F	54	STI	3 mth	STI in remission	46,XX,t(9;22)(q34;q11) 0/50
Pt 6	M	30	STI	3 mth	STI in remission	46,XY,t(9;22)(q34;q11) 3/50
Pt 9	F	67	STI	3 mth	STI in remission	46,XX,t(9;22)(q34;q11) 0/50
Pt 11	F	44	STI	3 mth	STI in remission	46,XX,t(9;22)(q34;q11) 0/100
Pt 17	M	33	none	at diagnosis	CP	46,XY,t(9;22)(q34;q11)
			STI	3 mth	STI in remission	46,XY,t(9;22)(q34;q11) 5/55
Pt 22	F	32	none	at diagnosis	CP	46,XX,t(9;22)(q34;q11)
			STI	3 mth	STI in remission	46,XX,t(9;22)(q34;q11) 0/50
Pt 21	M	79	none	at diagnosis	CP	46,XY,t(9;22)(q34;q11)
			Hydroxyurea	11 mth	AP	
			Hydroxyurea	13 mth	BC	

### 3.2.2 Real time PCR

RNA was extracted from normal and control samples with TRIZOL reagent as described in chapter 2. RNA was then DNase treated using the DNA-free reagent (Ambion) and cDNA synthesis was carried out as detailed in chapter 2 using the Ambion RETROscript kit.

To account for differences in input cDNA Beta-2-microglobulin ( $\beta 2M$ ) expression was examined with a pre-developed assay (Applied Biosystems, Foster City, CA). *hTERT* and *c-Myc* were also quantified using pre-developed assay reagents (Applied Biosystems). For confirmation of *hTERT* expression data an alternative assay amplifying a different region of the gene was used (Assays on Demand, Applied Biosystems). *TEP1*, tankyrase and *PinX1* expression were also quantified using Assays on Demand (Applied Biosystems). Primers and Taq-man probes for *hTR* and *TRF2* mRNA were designed using Primer Express 2.0 software (Applied Biosystems). Primers and probe for *TRF1* were as previously described (Yamada *et al.* 2002). These primer and probe sets were synthesized by MWG-Biotech (Milton Keynes, UK). Sequences of the oligonucleotides and probe for *hTR*, *TRF1* and *TRF2* are detailed below:

#### *hTR*:

Forward primer, 5'-GCTGTTTTTCTCGCTGACTTTCA-3',

Reverse primer, 5'-GCAGCTGACATTTTTTGTTTGCT-3', and

Probe, 5'-FAM-TGCCGCCTTCCACCGTTCATTCT-TAMRA-3'.



*TRF1:*

Forward primer, 5'-GCAACAGCGCAGAGGCTATTATT

Reverse primer, 5'-AGGGCTGATTCCAAGGGTGTA

Probe, 5'-FAM-TCCAGTCTAACAGCTTGCCAGTTGAGAACG-TAMRA-3'

*TRF2:*

Forward primer, 5'-ACCAGGGCCTGTGGAAAAG-3',

Reverse primer, 5'-GCACCAGACAGAGTCTTGAAAGC-3' and

Probe, 5'-FAM-AGAACCCGCAAGGCAGCTACGGAAT-TAMRA-3'.

A reverse transcriptase negative control was included for each assay to rule out any genomic DNA contamination. *GAPDH* (Applied Biosystems, Assays on Demand) was used as an alternative reference gene.

### 3.2.3 Quantification of *hTERT* transcript variants

cDNA from patient and control CD34<sup>+</sup> cells was PCR amplified as described in Chapter 2. A single primer set that would generate a product containing the deleted regions of the different *hTERT* transcripts was used (Drummond *et al.* 2005). The primer sequences were as follows: hT2164F: 5'-GCCTGAGCTGTACTTTGTCAA-3' and hT2620R: 5'-CGCAAACAGCTTGTTCTCCATGTC-3' with an annealing temperature of 64°C. The sequence amplified with these primers was confirmed using cycle sequencing. The control gene *β2M* was also amplified from the samples using the following primers designed using the Primer3 website (Rozen and Skaletsky 2000): *β2M*-F: 5'-GGCATTCTGAAGCTGACA-3' and *β2M*-R: 5'TCCCCCAAATTCTAAGCAGA-3' with an annealing temperature of 62°C. The

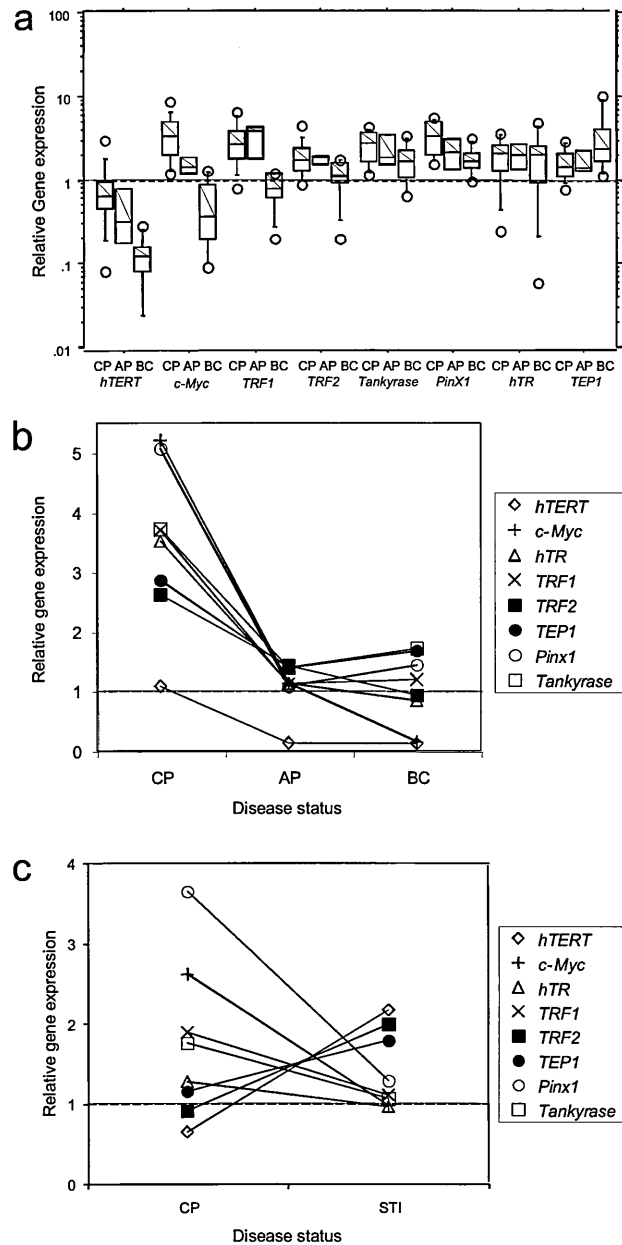
PCR products were then quantified using the Agilent Bioanalyser as described in Chapter 2.

### **3.3 Results**

#### **3.3.1 Real Time PCR analysis of Telomerase subunits**

##### **3.3.1.1 *hTERT***

Following data collection and analysis using Gene Amp 5700 SDS software the expression data of each gene was normalized to the endogenous reference  $\beta 2M$ . The normalized gene expression ( $\Delta ct$ ) of patients was related to the average  $\Delta ct$  of controls and the relative gene expression was calculated ( $2^{-\Delta \Delta ct}$ ). Statistically significant differences in gene expression between patient groups were identified using Graph Pad Instat 3. This analysis revealed that the expression levels of *hTERT* were reduced in the CD34<sup>+</sup> cells of patients with CP CML relative to the CD34<sup>+</sup> cells of normal individuals in 8 of 12 patients,  $P=.0387$ . *hTERT* was significantly down-regulated in the CD34<sup>+</sup>/blast cell fraction in 2 of 3 patients with AP CML and 7 of 7 patients with BC CML ( $P=.0017$ ). Fig 3.1a shows a progressive decrease of *hTERT* expression levels during CML disease evolution. Median expression levels of the different patient groups are shown in Table 3.2



**Fig 3.2 Relative gene expression of telomerase and associated genes.** CP= chronic phase AP= Accelerated phase BC=blast crisis STI= patients treated with imatinib. **(a)** Data from all CML patients. The data is shown on a logarithmic scale. Each box represents the expression of one gene, relative to controls, in one of the three groups of patients. The dashed line represents the approximate value of controls. **(b)** Data from a single patient during disease progression. Each line represents one gene and each symbol represents the expression level for that gene. The dashed line represents the approximate level of controls. **(c)** Data from a single patient following successful treatment with imatinib. Each line represents one gene and each symbol represents the expression level for that gene. The dashed line represents the approximate level of controls.

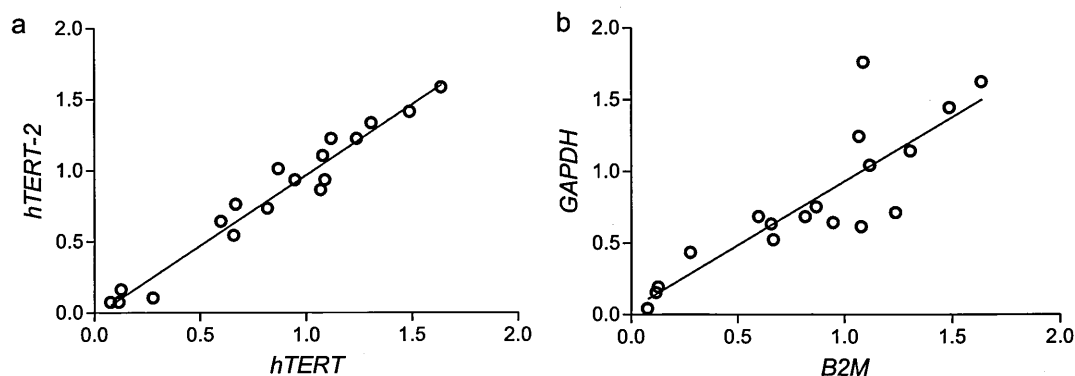
**Table 3.2 Relative gene expression ratios of telomerase and associated genes in CML.**

Control n=8, CP n=12, AP n=3, BC n=7, Imatinib n=7

Gene	Disease Status	Range	Median
<b><i>hTERT</i></b>	Control	0.67-1.31	1.08
	Chronic Phase	0.08-3.00	0.65
	Accelerated Phase	0.13-0.95	0.32
	Blast Crisis	0.01-0.28	0.12
	Imatinib	0.1-4.00	1.64
<b><i>hTR</i></b>	Control	0.70-1.66	0.95
	Chronic Phase	0.24-3.52	2.02
	Accelerated Phase	1.13-2.91	1.99
	Blast Crisis	0.06-4.98	2.02
	Imatinib	0.50-1.24	0.84
<b><i>TEPI</i></b>	Control	0.45-1.77	0.99
	Chronic Phase	0.76-2.86	1.43
	Accelerated Phase	1.21-2.53	1.39
	Blast Crisis	1.13-10.46	2.42
	Imatinib	0.89-1.78	1.47
<b><i>c-Myc</i></b>	Control	0.53-2.35	0.88
	Chronic Phase	1.20-8.76	3.34
	Accelerated Phase	1.13-2.02	1.42
	Blast Crisis	0.09-1.27	0.37
	Imatinib	0.48-1.40	0.98
<b><i>PinX1</i></b>	Control	0.70-1.18	0.99
	Chronic Phase	1.55-5.52	3.31
	Accelerated Phase	1.08-3.44	2.13
	Blast Crisis	0.97-3.14	1.73
	Imatinib	0.50-1.50	1.23
<b><i>TRF1</i></b>	Control	0.65-1.53	0.99
	Chronic Phase	0.78-6.45	2.69
	Accelerated Phase	1.12-4.49	3.79
	Blast Crisis	0.20-1.22	0.80
	Imatinib	0.68-1.54	1.06
<b><i>TRF2</i></b>	Control	0.82-1.43	0.97
	Chronic Phase	0.89-4.46	1.70
	Accelerated Phase	1.43-1.93	1.81
	Blast Crisis	0.20-1.80	1.13
	Imatinib	0.77-2.12	1.26
<b><i>Tankyrase</i></b>	Control	0.74-1.35	1.01
	Chronic Phase	1.13-4.30	2.73
	Accelerated Phase	1.40-3.94	1.85
	Blast Crisis	0.67-3.42	1.72
	Imatinib	0.75-1.63	1.06

To further illustrate the decrease of *hTERT* expression, samples were available from CP, AP and BC from one of the patients included in this study (pt21) giving an insight into the dynamics of telomerase gene expression in a single case during disease progression. In serial samples from this patient it can be seen that expression levels of *hTERT* in decrease with disease progression (Fig 3.2b).

In order to validate the above data results were confirmed using an alternative assay amplifying a different region of the *hTERT* transcript in 18 samples, from which there was sufficient material. Results showed a high level of correlation, Spearman  $r=0.9716$ ,  $P<0.0001$ , Fig 3.3a.



**Fig 3.3 Comparison of alternative gene expression assays for determination of *hTERT* expression.** Trend lines were calculated using linear regression statistics. (a) Comparison of two applied biosystems gene expression assays for *hTERT*. Expression ratios of *hTERT* expression are represented by open circles. *hTERT*= Pre developed assay reagent 4319447F. *hTERT*-2= Assay on Demand Hs00162669\_m1. (b) Comparison of  $\beta 2M$  and *GAPDH* control genes for determination of *hTERT* expression. Expression ratios of *hTERT* are represented by open circles.

To further verify the results *hTERT* expression data was confirmed in 18 samples, using *GAPDH* as a control gene. These results correlated well with the expression of *hTERT* using  $\beta 2M$  as a control (Spearman  $r=0.855$ ,  $P=.0004$ ), Fig 3.3b. The same test was

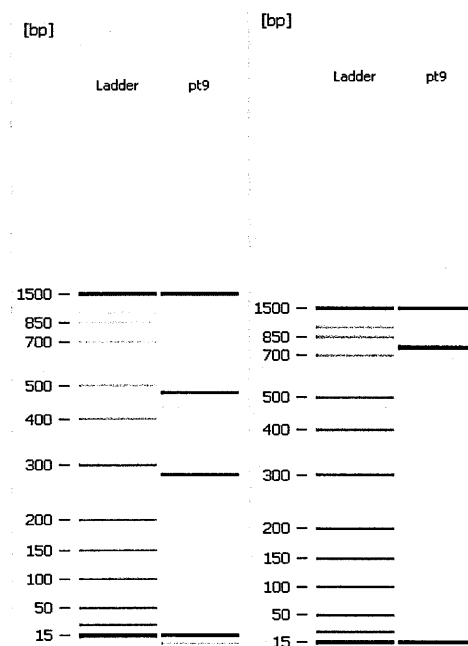
performed with *GAPDH* as a control for expression of the other telomerase associated genes and results showed high correlation.

### **3.3.1.2 Splicing of *hTERT* RNA**

The *hTERT* transcript has four different splice variants that represent an additional level of gene regulation. The  $+\alpha+\beta$  transcript is full length functional *hTERT*.  $-\alpha+\beta$  is a dominant negative inhibitor of *hTERT* activity while the  $+\alpha-\beta$  and  $-\alpha-\beta$  transcripts are inactive truncated proteins. Quantification of *hTERT* splice variants in CML will give insight into the mechanisms of *hTERT* regulation.

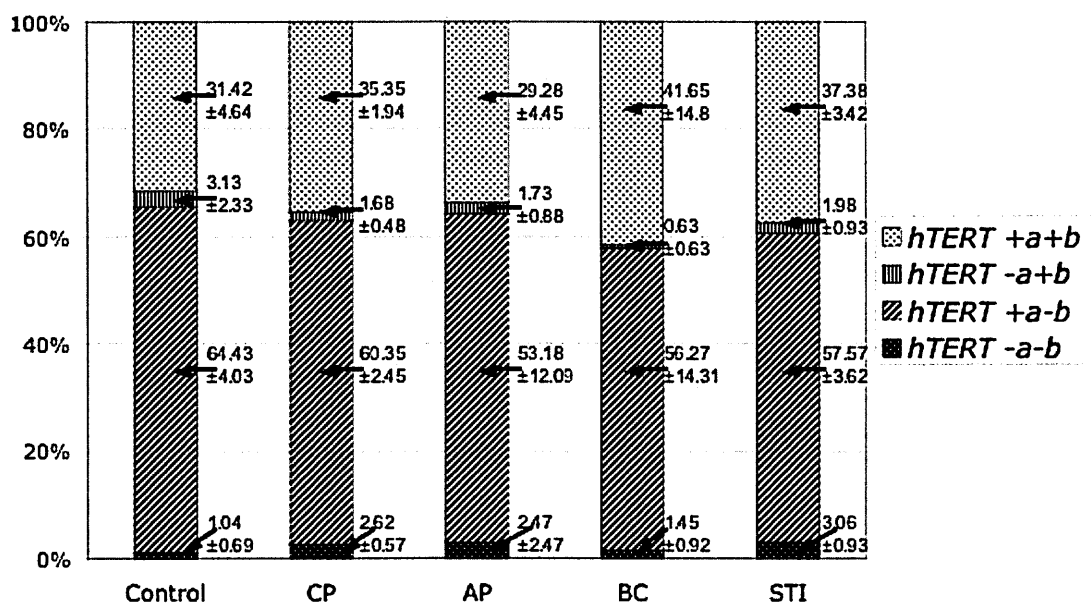
The four *hTERT* splice variants can be amplified in a single PCR amplification. However, it is not possible to resolve all fragments using standard agarose gel electrophoresis. Using the Agilent Bioanalyzer DNA 1000 assay it has been possible to resolve and quantify *hTERT* splice variant PCR products in the CD34<sup>+</sup> cells of CML patients and normal controls. The microfluidics based electrophoresis platform produces an electropherogram for each sample and then converts the electropherogram into a gel like image. DNA is quantified using an internal standard. Quantities of the individual transcript variants are expressed as a percentage of total *hTERT* expression. An example of a case showing all four *hTERT* splice variants is shown in Fig 3.4.

All samples in this study expressed the  $+\alpha+\beta$  and  $+\alpha-\beta$  *hTERT* splice variants. The  $-\alpha+\beta$  and  $-\alpha-\beta$  variants were expressed at low levels but not in all samples.  $-\alpha-\beta$  was expressed in 2 of 8 controls, 9 of 12 CP, 1 of 3 AP and 2 of 7 BC.  $-\alpha+\beta$  was expressed in 3 of 8 controls, 7 of 9 CP, 2 of 3 AP and 1 of 7 BC.



**Fig 3.4 Agilent Bioanalyzer gel like image of *hTERT* splice variants.** The internal markers are shown as purple or green bands on the gel and the DNA fragments are visualised between these markers. In lane 2 four bands can be seen corresponding to the four splice variants. One band is visible in lane 4 representing the  $\beta 2M$  control amplification.

It can be seen in Figure 3.5 that these results indicate a shift in the splicing patterns of *hTERT* in the  $CD34^{+}$  cells of patients with CML. The proportion of  $+\alpha-\beta$  is decreased in CML patients in all stages of disease and the proportion of  $+\alpha+\beta$  transcripts is increased. However, these changes do not reach statistical significance. The levels of  $-\alpha+\beta$  and  $-\alpha-\beta$  transcripts were not significantly different between patients and normal controls.



**Figure 3.5 *hTERT* transcript expression.** Splice variant levels in each disease stage are shown as the percentage that each variant contributes to the total. The figures represent the mean for each transcript in each group with the standard error of the mean shown below. CP= chronic phase AP= Accelerated phase BC=blast crisis STI= patients treated with imatinib.

### 3.3.1.3 *hTR*

The *hTR* gene encodes the telomerase RNA template from a single exon gene. This means that any real time PCR assay for *hTR* will amplify cDNA and any contaminating genomic DNA in the sample. All RNA samples included in this study were DNase treated before cDNA synthesis. This should remove any contaminating genomic DNA from the samples. To ensure that this was the case a reverse transcriptase negative control was included and this was always negative.

Real time PCR and data analysis of *hTR* expression showed that median levels of *hTR* were increased in the CD34+ cells of patients in CP relative to normal controls. However, there was a wide range in the expression levels and two patients had expression levels below the range of controls (ratio 0.52 and 0.24). With the exception



of one patient in BC with a marked decrease in *hTR* levels (ratio 0.06), levels of expression were relatively constant during disease progression. These results suggest that there is a trend towards increased *hTR* expression in CML, however, any differences in *hTR* gene expression levels did not reach statistical significance ( $P=.1256$ ).

#### **3.3.1.4 *TEP1***

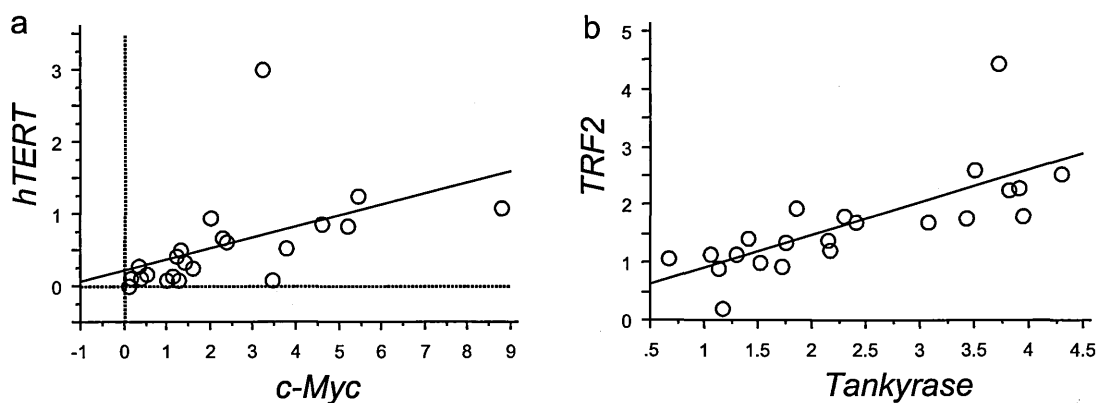
*TEP1* is the third telomerase holoenzyme subunit included in this study. The expression levels of *TEP1* were increased relative to normal controls in CP, but not to a statistically significant degree. *TEP1* expression levels were highest in BC CML and significantly higher than normal controls ( $P=.0037$ ). This is the only telomerase related gene to show a significant increase above normal control expression levels in BC CML.

### **3.3.2 Real time PCR Analysis of Telomerase Regulators**

#### **3.3.2.1 *c-Myc***

*c-Myc* activates expression of telomerase by directly binding the *hTERT* promoter. *c-Myc* expression levels were high relative to controls in CP CML but, like *hTERT*, decreased with disease progression. In the majority of patients in BC expression levels of *c-Myc* were lower than that of normal CD34<sup>+</sup> cells. *c-Myc* expression in BC was significantly lower than CP and AP ( $P=.0001$ ).

The action of *c-Myc* to increase transcription of telomerase indicates that a correlation in the expression of these two genes is likely. Using the Spearman rank correlation test to compare *hTERT* and *c-Myc* expression in all patient samples it can be seen in Figure 3.6a that *c-Myc* expression levels correlated positively with *hTERT* expression in all stages of CML (Spearman  $r=0.744$ ,  $P=.0007$ ). This result is further enforced by the fact that expression of both *hTERT* and *c-Myc* decrease in a single patient (pt21) during disease progression (Fig 3.2b).



**Figure 3.6 Correlation of gene expression levels in telomerase associated genes.** Trend lines were calculated using linear regression statistics. (a) *hTERT* and *c-Myc* expression levels correlate in all stages of disease in CML. Spearman  $r=0.744$   $P=.0007$ . (b) Correlation between *TRF2* and *Tankyrase* expression levels in CML patients in all stages of CML. Spearman  $r=0.859$   $P=<.0001$ .

### 3.3.2.2 *PinX1*

*PinX1* inhibits telomerase enzyme activity by binding the catalytic subunit to stop binding of the RNA template. Expression levels of *PinX1* were high, relative to controls, in the majority of patients in CP and AP. Median levels of *PinX1* expression decrease with disease progression. However, the majority of patients in BC had higher *PinX1* expression levels than controls and these differences were statistically significant ( $P=.0037$ ).

### **3.3.4 Real Time PCR analysis of Telomeric-Associated Genes**

#### **3.3.4.1 TRF1**

The telomere repeat binding factor *TRF1* restricts access of telomerase to the telomere repeats. Expression of *TRF1* was increased in the majority of patients in CP, relative to normal controls ( $P=.0015$ ). In contrast, *TRF1* expression levels were reduced, relative to normal controls, in the majority of patients in BC.

#### **3.3.4.2 TRF2**

The main function of *TRF2* is telomere end protection. Expression levels of *TRF2* were increased in the CD34<sup>+</sup>/blast cells of the majority of CML patients in CP and AP. This trend was not maintained during BC and levels were in the range of controls in the majority of these patients.

#### **3.3.4.3 Tankyrase**

*Tankyrase* positively regulates telomere length by interacting with *TRF1*. Expression levels of *tankyrase* were highest in patients with CP and AP CML. Analysis by the Kruskal-Wallis test revealed that *Tankyrase* expression in the three patient groups was significantly higher than controls,  $P=.0028$ . Although *tankyrase* has no known effect on the transcription of *TRF2*, or vice versa, it is of interest to note that the expression levels of these two factors correlated in all stages of disease in CML patients (Spearman  $r=0.859$ ,  $P<.0001$ ). This is illustrated in Figure 3.6b.

### ***3.3.5 Expression of telomerase and telomerase-associated genes in patients treated with imatinib***

Patients treated with imatinib can reach cytogenetic remission in as little as three months. If all cells are of normal karyotype then samples from these patients could be considered as haematologically normal, providing an internal control for expression data in these patients.

#### ***3.3.5.1 hTERT***

The expression levels of *hTERT* were increased in the CD34<sup>+</sup> cells of seven CP CML patients in remission following successful treatment with imatinib, relative to untreated CP patients. Furthermore, *hTERT* expression was not significantly different from normal individuals ( $P=.1520$ ). Two of the patients included in this analysis were also sampled in CP at diagnosis. In these two patients it can be seen that, after treatment with imatinib, *hTERT* expression is returning to levels similar to those of normal CD34<sup>+</sup> cells. The data from one of these patients (pt17) is shown in Figure 3.2c to illustrate this point.

#### ***3.3.5.2 Splicing of hTERT RNA***

Expression of the telomerase transcript variants in patients treated with imatinib is summarised in Fig 3.5.  $-\alpha-\beta$  was expressed in 5 of the 7 patients treated with imatinib and  $-\alpha+\beta$  was expressed in 4 of 7. Similar to CP patients the proportion of  $+\alpha-\beta$  is decreased and the proportion of  $+\alpha+\beta$  transcripts is increased when compared to the normal control group. Figure 3.5 shows that expression of *hTERT* splice variants in

patients treated with imatinib is more similar to CP patients than to normal controls. The differences in  $+\alpha+\beta$  and  $+\alpha-\beta$  splice variants are not statistically different between the STI, CP and control groups.

#### **3.3.5.3 *hTR* and *TEPI***

The median expression levels of *hTR* in these seven patients were reduced as compared to untreated CP patients. The same pattern of gene expression was also observed in samples taken from two patients pre-and post- treatment with imatinib (Fig 3.2c). Overall, *TEPI* expression was decreased in patients treated with imatinib and furthermore the expression levels of *TEPI* fell within the range observed in normal controls.

#### **3.3.5.4 *Telomerase regulators***

Median expression levels of the telomerase regulators *c-Myc* and *PinX1* were reduced in patients following successful treatment with imatinib when compared to untreated CP patients. As illustrated in Figure 3.2c, this pattern is again echoed in patients where samples have been obtained pre- and post- treatment with imatinib.

#### **3.3.5.5 *Telomerase associated genes***

In correlation with the above data median expression levels of *TRF1*, *TRF2* and *tankyrase* were reduced in the group treated with imatinib when compared to untreated CP patients. As can be seen in Table 3.2 the levels of expression of *TRF1* fell within the range of normal controls.

### 3.4 Discussion

The expression levels of the components of telomerase, its regulators and several telomeric associated proteins have been evaluated in the CD34<sup>+</sup>/blast cell populations of 22 CML patient samples as compared to the CD34<sup>+</sup> cell population of eight healthy individuals. Expression levels of *hTR*, *hTERT* and *TEP1*, (the three major subunits of telomerase), *TRF1*, *TRF2* and *tankyrase*, (telomeric-associated proteins) and *c-Myc* and *PinX1*, (positive and negative regulators of telomerase, respectively), have been determined by real-time quantitative PCR analysis.

The discovery that *hTERT* is down regulated in the CD34<sup>+</sup> cells of patients with CML is in contrast to earlier studies investigating telomerase activity and protein levels (Broccoli *et al.* 1995; Tatematsu *et al.* 1996; Ohyashiki *et al.* 1997; Bitisik *et al.* 2000; Verstovsek *et al.* 2003). The finding of the current study is robust given the levels of validation of the result: Two different real time PCR assays for telomerase were used with two different control genes, each time showing the same result. The previous studies detailed above measure telomerase enzyme activity rather than *hTERT* expression in unfractionated bone marrow samples (Verstovsek), bone marrow mononuclear cells (Ohyashiki) or peripheral blood mononuclear cells (Ohyashiki, Bitisik, Tatematsu) of CML patients. In the study by Ohyashiki *et al.* (Ohyashiki *et al.* 1997) bone marrow mononuclear cells or peripheral blood samples from 33 CP patients were shown to have telomerase activity above background levels measured in normal peripheral blood. It was also noted that telomerase activity in mononuclear cells from 21 patients in BC had a significant increase in telomerase activity when compared to both CP patients and normal control peripheral blood cells. Similar findings were presented in the studies by Tatematsu and Bitisik who compared the CML samples to

peripheral blood mononuclear cells. However, the mononuclear cells of BC CML patients will have a higher percentage of blast cells (expressing telomerase) than CP patients and the difference in telomerase activity between these patient groups could be explained by the increased number of immature cells present in the BC samples. Similarly, mononuclear cells from CP CML will have more blast cells present than normal control peripheral blood. In the present study *hTERT* expression in normal CD34<sup>+</sup> cells has been compared with CML CD34<sup>+</sup>/blast cells, i.e. comparing like with like. Therefore, the differences in telomerase activity between this and previous studies may be a reflection of the cell types studied. In fact, this is consistent with a study by Li *et al.* (Li *et al.* 2000) which shows a linear correlation between telomerase activity and the logarithm of bone marrow blast percentages in CML. Additionally, Gene expression profiles from peripheral blood and bone marrow samples in CML have significantly different gene expression profiles, authors identified 459 genes that were differentially expressed between peripheral blood and bone marrow samples from CML patients highlighting the importance of analysing the correct cell type (Nowicki *et al.* 2003).

A paper by Drummond *et al.* (Drummond *et al.* 2005) supports the findings of this study. They showed that *hTERT* expression in CD34<sup>+</sup> cells of CP CML patients is not significantly different from expression in CD34<sup>+</sup> cells of normal controls. It was also shown that telomerase activity, when corrected for the decreased number of cells in G<sub>0</sub> in CP, is not significantly increased in CML when compared with controls. This suggests that the small differences in telomerase activity between these samples are a reflection of cell cycle status rather than a real increase in telomerase activity. A recent investigation by Gerber *et al.* further confirms the result of reduced *hTERT* expression in CML (Gerber *et al.* 2011). They isolated CML stem cells and compared then with

stem cells of normal controls and showed that *hTERT* expression was reduced in the patient samples when compared to normal control stem cells and appeared to decrease with disease progression.

It may be suggested that this decrease in telomerase is a result of the *BCR/ABL* fusion gene in CML. In fact, it has been shown that antisense inhibition of *BCR/ABL* or *c-Abl* increases *hTERT* levels (Bakalova *et al.* 2004). Therefore, the presence of the *BCR/ABL* fusion in CML may contribute to the down regulation of *hTERT* observed in the  $CD34^+$ /blast cells of the CML patients in this study.

An additional level of gene regulation of *hTERT* is the alternative splicing of the gene. Investigation of the proportions of the four alternative splice variants of *hTERT* was carried out. Results indicate that the relative proportion of full length active  $+\alpha+\beta$  transcripts is increased in CML while the proportion of inactive  $+\alpha-\beta$  is decreased, although this does not reach statistical significance. This indicates that while the overall expression levels of *hTERT* are decreased in the  $CD34^+$  cells of CML patients a shift in splicing patterns could allow a higher proportion of  $+\alpha+\beta$  transcripts to be active and therefore maintain telomere length above the critical level allowing the leukaemic cells to proliferate. Drummond *et al.* similarly noted an increase in the levels of the  $+\alpha+\beta$  transcripts at the expense of the  $+\alpha-\beta$  in CP CML as compared to normal controls (Drummond *et al.* 2005).

The data in the present study shows a trend for increasing *hTR* levels in the  $CD34^+$  cells from the majority of CML patients in all phases (however, two CP patients have *hTR* expression levels below the range of normal controls). Levels of *hTR* were also investigated by Drummond *et al.* (Drummond *et al.* 2005) and a significant decrease in the expression of *hTR* was noted in some CP CML patients. In the majority of patients



in the present study the changes in expression levels of *hTR* did not correlate with the changes in expression levels observed for *hTERT* and any changes in *hTR* expression did not reach statistical significance. Similar observations have been made in other malignancies including AML and thyroid cancer (Xu *et al.* 1998; Hoang-Vu *et al.* 2002).

The expression levels of the telomerase enzyme component *TEP1* increased with disease progression of CML. This is the only telomerase component gene to be significantly up regulated in BC CML. *TEP1* expression has also been shown to be increased in and breast cancer where it has prognostic value. Salhab *et al.* (Salhab *et al.* 2008) showed that expression levels of *TEP1* could predict overall survival in breast cancer. However, it has been shown that *TEP1* is not an essential protein of the telomerase complex and that telomerase can function in the absence of *TEP1* (Koyanagi *et al.* 2000; Liu *et al.* 2000). However, *TEP1* is required for full enzyme activity and is expressed at relatively constant levels in human tissues and cell lines (Chang *et al.* 2002). The expression of *hTERT* is the limiting factor for the function of telomerase and *TEP1* may be expressed in cells with no effect and only used for assembly of the telomerase complex when *hTERT* is expressed.

Expression levels of *c-Myc* were increased in CP CML in relation to normal controls and significantly decreased with disease progression. Additionally expression levels of *c-Myc* positively correlated with expression of *hTERT*. An increase in *c-Myc* has previously been demonstrated by microarray analysis in the CD34<sup>+</sup> cells of CP CML patients and this is in agreement with the current study (Diaz-Blanco *et al.* 2007). It is known that *c-Myc* positively regulates *hTERT* expression and a significant association between increased *c-Myc* expression and increased *hTERT* expression has been

demonstrated in several cancers such as breast, prostate and cervical cancers (Bieche *et al.* 2000; Latil *et al.* 2000; Sagawa *et al.* 2001). Furthermore, telomerase activity is inhibited in leukaemia cells treated with anti-sense oligonucleotides targeting *c-Myc* (Fujimoto and Takahashi 1997). It could therefore be concluded that the decrease in *hTERT* expression in the CD34<sup>+</sup>/blast cells of BC CML patients may be directly caused by the reduction in expression of *c-Myc* in these patients.

Expression of the telomerase inhibitor *PinX1* was significantly increased in the majority of CML patients. In addition to its role in telomerase enzyme inhibition it has recently been shown that *PinX1* may act through *c-Myc* to control expression of *hTERT*. Wang *et al.* demonstrated that transfection of *PinX1* into *PinX1* negative gastric cancer cells reduced expression of *c-Myc* and telomerase activity (Wang *et al.* 2010). Furthermore, *PinX1* RNAi experiments resulted in increased expression of *c-Myc*. Therefore, it can be suggested that in the CML patients included in this study the presence of elevated *PinX1* expression above normal control levels could contribute to the resulting decrease in *c-Myc* expression which in turn leads to decreasing *hTERT* expression.

*TRF1* and *TRF2* expression levels were increased in the CD34<sup>+</sup>/blast cells of CP patients and expression of both genes decreased with disease progression. *TRF1* inhibits the action of telomerase at the telomeric region to control telomere length (van Steensel and de Lange 1997). Increased TRF1 leads to telomere shortening. The increased expression levels of *TRF1* in CP and AP may contribute to the telomere shortening observed in CML. Both TRF1 and TRF2 restrict elongation of telomeres so a decrease in expression of these genes with disease progression suggests that in BC telomerase would be able to access the telomeres and the low levels of telomerase would be able to maintain telomere length above the critical level. High expression

levels of *TRF2* in human primary cells have been shown to prevent or delay the signalling of critically shortened telomeres to induce senescence (Karlseder *et al.* 2002). The presence of *TRF2* expression in CML could be preventing the signalling of short telomeres in the patients allowing the leukaemic cells to continue to proliferate with critically short telomeres.

*Tankyrase* expression levels were significantly increased in the CD34<sup>+</sup> cells of CML patients in all stages of disease. This is in agreement with previous findings in breast cancer where expression of *Tankyrase* is increased in cancerous tissue when compared to the adjacent normal tissue (Poonepalli *et al.* 2008). Overexpression of *Tankyrase* in telomerase-positive human cells inhibits the action of TRF1 and results in elongation of telomeres (Smith and de Lange 2000). If this is the case in CML then it could be concluded that increased *Tankyrase* expression in patients allows optimal telomerase elongation of telomeres by the low levels of telomerase present in the patient samples. The correlation noted in the expression levels of the telomere binding proteins *TRF2* and *Tankyrase* suggests that the parallel up-regulation of these genes could contribute to telomere maintenance in CML.

Seven patients successfully treated with imatinib were included in this study. Expression levels of *hTERT* were increased in the CD34<sup>+</sup>/blast cells of these patients compared with untreated CP patients. If *hTERT* expression is increased then it would be expected that telomere length would also increase. Indeed, it has been reported that peripheral blood leukocyte telomere length increases in CML patients after long term treatment with imatinib (Brummendorf *et al.* 2003).

The expression levels of *hTR*, *c-Myc*, *TRF1*, *TRF2*, *PinX1* and *Tankyrase* in the seven patients included in the present study were markedly reduced as compared to untreated CP CML patients and were not significantly different from normal controls. Furthermore, the expression levels of *TEP1* and *TRF1* fell within the range observed in normal controls. These changes were also observed in serial samples taken from two patients pre- and post- treatment with imatinib. These results reflect the restoration of haematopoiesis towards normality as the leukaemic cells are depleted during treatment. Expression levels of the telomerase associated genes are returning to the normal range in the patients that have undergone successful treatment with imatinib. This is in agreement with a gene expression profiling study of CD34<sup>+</sup> cells by Neumann *et al.* (Neumann *et al.* 2005) investigating patients in remission following treatment with imatinib. Neumann *et al.* demonstrate that most genes have unchanged expression in patients in cytogenetic remission after treatment when compared to normal controls. The patients in the present study were treated for three months, while in the investigation by Neumann *et al.* patients had undergone treatment for at least eleven months. It is possible that with longer periods of treatment expression of all genes investigated in the present study will fall within the normal range in these patients.

This data challenges the view that *hTERT* is always increased in cancer. The reduced expression of *hTERT* in the CD34<sup>+</sup> cells of CML patients suggests that levels of the enzyme in these cells would be reduced with disease progression. Disease progression of CML is associated with a decrease in telomere length. It could therefore be suggested that decreased expression of *hTERT* is contributing to the shortened telomeres observed in CML. Expression of *hTERT* is likely to be reduced by the reduction of *c-Myc* expression, increased *PinX1* expression and by the presence of the *BCR/ABL* fusion gene. The presence of *TRF2* may prevent shortened telomeres being recognised

as double strand breaks and allow the leukaemic cells to keep dividing with critically short telomeres which will promote genetic instability and which can in turn lead to further genomic aberrations that are associated with disease progression.

## ***Chapter 4***

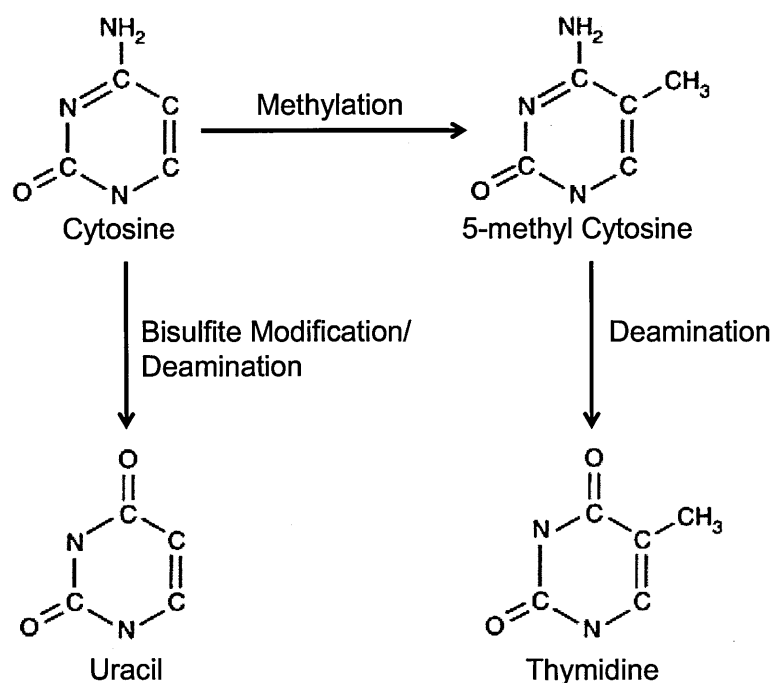
# ***Causes of deregulated hTERT expression in Chronic Myeloid Leukaemia***

## **4.1 Introduction**

### **4.1.1 Control of *hTERT* gene expression by methylation**

DNA methylation refers to the modification of a cytosine residue at CpG dinucleotide sites in the genome (Ehrlich and Wang 1981). The cytosine is methylated at position C5 of the CpG site and is referred to as 5-methylcytosine (Ehrlich and Wang 1981). Methylation is carried out by DNA methyltransferase enzymes (Hsu *et al.* 1999). CpG sites occur in clusters along the mammalian genome and are named CpG islands (Bird 1986). These CpG islands are estimated to be associated with the promoter regions of 50-60% of human genes (Cross and Bird 1995; Ewing and Green 2000). Methylation of CpG sites is associated with lack of expression of a gene. Methylation inhibits transcription factor binding in the promoter region of a gene leading to repression of transcription (Robertson 2005). The majority of CpG sites are not methylated and CpG islands of normal mammalian cells are in general not methylated (Warnecke and Bestor 2000). The primary role of methylation in the genome is to protect against transcriptional activation of transposon promoters by methylating these regions (Bestor 1998). Additionally, DNA methylation is essential for normal development and is involved in X-chromosome inactivation and imprinting (Li *et al.* 1993; Panning and Jaenisch 1998). Imprinting is the phenomenon of differential expression of the maternal and paternal alleles of a gene and is controlled by DNA methylation (Bartolomei and Tilghman 1997). A well characterised example of an imprinted gene is *H19* which expresses a non-coding RNA with tumour suppressor functions (Yoshimizu *et al.* 2008). Methylation of the promoter region of the paternal allele of *H19* ensures that expression is exclusively controlled through the maternal allele (Zhang and Tycko 1992; Jinno *et*

*al.* 1996). This means that the paternal allele of *H19* is always methylated and not expressed whereas the maternal allele is always unmethylated and fully active.



**Figure 4.1 DNA methylation and Modification.** Biochemical changes in the structure of Cytosine are shown when the base undergoes methylation and subsequent deamination or bisulfite modification. Figure adapted from Singal and Ginder 1999.

Aberrant methylation has been proposed to contribute to carcinogenesis (Warnecke and Bestor 2000). Increased hypomethylation of primary human cancer cells compared to normal tissues was first demonstrated by Feinberg and Vogelstein (Feinberg and Vogelstein 1983). This loss of methylation is thought to be a result of demethylation of repetitive transposon elements of the genome and subsequent activation of these elements may contribute to genetic instability in tumour cells (Yoder *et al.* 1997). Although cancer cells undergo general hypomethylation there is also hypermethylation of the CpG islands of specific genes resulting in gene silencing (Issa 2004). Genome wide methylation analysis has revealed that CpG island methylation is present in both low and high grade tumours and it appears that the patterns of methylation are not



random with some CpG islands being preferentially methylated (Costello *et al.* 2000). Indeed, CpG island methylation has been associated with reduced expression of several tumour suppressor genes. Methylation of the promoter CpG island of the Cyclin Dependant Kinase inhibitor CDKN2A has been identified in many cancers including lung carcinoma and gastric carcinoma and this methylation is known to lead to decreased expression of the gene (Oue *et al.* 2003; Nakata *et al.* 2006). CDKN2A is a known tumour suppressor gene that controls cell cycle progression and methylation in T-cell leukaemia suppresses expression and is associated with disease progression in these patients (Nosaka *et al.* 2000). The retinoic acid receptor RARB is a ligand activated transcription factor that limits cell growth by regulating gene expression and decreased RARB is associated with carcinogenesis (Xu *et al.* 1994; Widschwendter *et al.* 1997; Xu *et al.* 1997). Methylation resulting in decreased expression of RARB has been identified in several malignancies including cervical cancer and acute promyelocytic leukaemia (Chim *et al.* 2003; Narayan *et al.* 2003).

The presence of methylation at a CpG site is associated with an increase in the rate of mutation. The cytosine residue of a CpG dinucleotide is vulnerable to deamination, which when not methylated, produces uracil and is repaired by the mismatch repair machinery of the cell (Holliday and Grigg 1993). However, deamination of 5-methylcytosine produces thymine which is a normal DNA base and therefore not recognised by the cell as an error and so is not repaired by the cells mismatch repair machinery (Holliday and Grigg 1993). As a result of this the CpG dinucleotide is under represented in the genome and the mutation rate of the 5-methylcytosines at these sites is at least 12 times higher than the normal transition rate (Sved and Bird 1990).

The *hTERT* CpG island has been identified and has been shown to be controlled by methylation. Devereux *et al.* assessed the *hTERT* promoter methylation status in normal tissue, immortal cells and cancer cell lines (Devereux *et al.* 1999). They identified methylation of the *hTERT* promoter in these samples. Although they did not identify a pattern of specific methylation that correlated with *hTERT* expression they demonstrated that demethylation of an *hTERT* negative cell line induced the cells to express *hTERT*. Interestingly, it has been shown that methylation of the *hTERT* promoter can be correlated with gene expression rather than gene silencing (Guilleret *et al.* 2002). In normal and tumour tissues *hTERT* expression was observed in those samples with hypermethylation of the *hTERT* promoter (Guilleret *et al.* 2002). However, it was later discovered that the *hTERT* CpG island can be extensively methylated and produce *hTERT* transcripts as long as levels of methylation around the transcription start site are low or absent (Zinn *et al.* 2007).

The status of methylation of the *hTERT* gene has been assessed in human cancers including gastric cancer, cervical cancer and B-cell lymphocytic leukaemia (Bechter *et al.* 2002; Gigeck *et al.* 2009; Iliopoulos *et al.* 2009). Hypermethylation was shown to be associated with low telomerase activity in B-cell lymphocytic leukaemia and in cervical cancer hypermethylation was associated with cervical oncogenesis progression (Bechter *et al.* 2002; Iliopoulos *et al.* 2009). However, in gastric carcinomas the *hTERT* promoter was more methylated in cancer than in normal gastric mucosa and this correlated with an increase in *hTERT* protein levels (Gigeck *et al.* 2009). Additionally, in colorectal cancers and cell lines increased *hTERT* expression correlates with hypermethylation of the *hTERT* promoter. This indicates that the role of promoter methylation in controlling gene expression of *hTERT* is complex and is unlikely to be the only factor contributing to control of gene expression.

#### **4.1.2 Control of *hTERT* expression: Promoter mutation**

As discussed in chapter 3 expression of *hTERT* is controlled by the binding of c-Myc and SP1 to the promoter of the gene. The *hTERT* core promoter is thought to be responsible for the transcriptional activity of the *hTERT* gene (Takakura *et al.* 1999). The promoter contains two E-boxes where c-Myc will bind and an SP-1 consensus motif where the general transcription factor SP-1 binds and these sites are required for activation of *hTERT* (Takakura *et al.* 1999). Mutations in these sites can significantly decrease activation of *hTERT* transcription indicating that mutation stops transcription factor binding (Kyo *et al.* 2000). The promoter also contains an Ets2 binding site. Ets binds the *hTERT* promoter at this site to transduce a signal from Epidermal Growth factor (EGF) to activate telomerase (Maida *et al.* 2002). Polymorphism of the Ets2 binding site has been correlated with a decrease in *hTERT* expression in non-small cell lung cancer (Hsu *et al.* 2006). The SNP is a T to C substitution at -245 bp from the transcription start site and those patients with the genotype C/C or T/C at this locus were shown to have lower telomerase activity than T/T homozygotes.

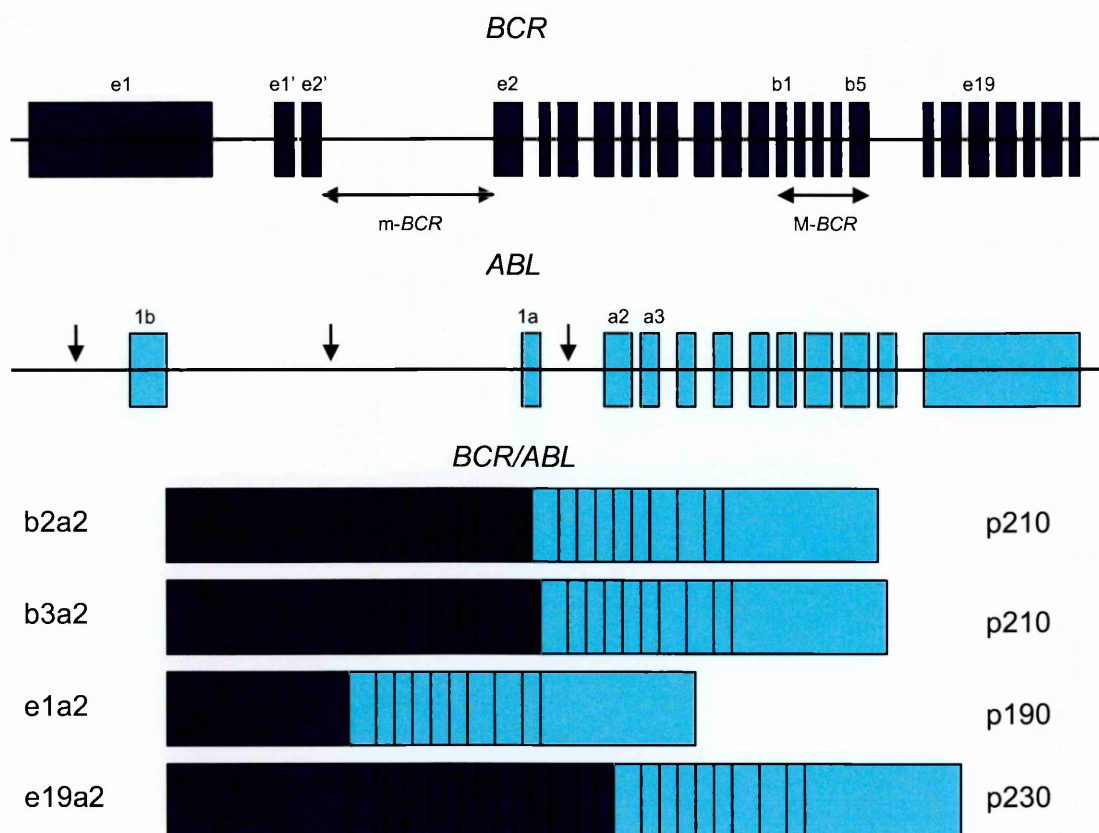
The *hTERT* promoter can be methylated and given the increased rate of mutation in methylated CpG sites it will be of interest to investigate the mutation status of the *hTERT* promoter in CML.

#### **4.1.3 *BCR/ABL* detection in CML**

The *BCR/ABL* t(9;22) reciprocal translocation is the hallmark genetic abnormality and diagnostic standard of CML. The breakpoints of both *BCR* and *ABL* vary between patients as shown in Fig 4.2. The majority of breakpoints of *BCR* occur in a 5.8kb

region termed the major breakpoint cluster region (M-*BCR*) (Groffen *et al.* 1984). M-*BCR* contains exons 12 to 16 of the *BCR* gene which historically were named b1 to b5 (Melo 1996). The *ABL* gene has two alternative first exons known as 1a and 1b and breakpoints in this gene occur either between exons 1a and 1b, downstream of exon 1a or upstream of exon 1b (Melo 1996). Most commonly *BCR* exon b2 or b3 is fused to *ABL* exon a2 upstream of *ABL* exon 1b giving rise to the p210 fusion protein seen in the majority of CML patients (Deininger *et al.* 2000). Sporadically *BCR* can also break within the minor breakpoint cluster region (m-*BCR*) between the two alternative exons e2' and e2 which gives rise to an e1a2 p190 fusion product (Chan *et al.* 1987). This fusion product can be observed in patients with acute lymphoblastic leukaemia and only in sporadic cases of CML (Verma *et al.* 2009). In rare cases of CML a third type of transcript is observed which is a product of a fusion between *BCR* exon 19 and *ABL* a2 (Saglio *et al.* 1990). This gives rise to a larger p230 fusion protein (Wilson *et al.* 1997).

Initial diagnosis of CML is based on the characteristic blood count showing overproduction of granulocytes (Hehlmann *et al.* 2007). Presence of the *BCR/ABL* fusion transcript is then confirmed by fluorescent in situ hybridisation and can also be detected by real time PCR (Hehlmann *et al.* 2007). Real time PCR has been used extensively for research purposes to monitor *BCR/ABL* transcripts in patients with CML (Hughes *et al.* 2006). Monitoring of *BCR/ABL* transcript levels by real time PCR in CML shows that *BCR/ABL* levels are significantly increased with disease progression (Elmaagacli *et al.* 2000). It has been shown that high levels in CP can predict a progression of disease to AP and BC (Gaiger *et al.* 1995). Additionally, the increase in *BCR/ABL* transcripts is an actual increase in mRNA in malignant cells and does not reflect an increase in levels of leukaemic cells (Gaiger *et al.* 1995). Measurement of *BCR/ABL* expression by Real time PCR has been introduced to routine testing for CML



**Figure 4.2 *BCR/ABL* breakpoint variants.** Exons are represented by coloured boxes. The alternative first and second exons of *BCR* are labelled *e1*, *e1'*, *e2* and *e2'* and the breakpoint regions are indicated with double headed arrows. The common breakpoints of *ABL* are indicated by arrows. The resulting fusions transcripts are shown with the molecular weight of the proteins.

patients treated with imatinib. Imatinib specifically inhibits the tyrosine kinase activity of *BCR/ABL* by competitively inhibiting the ATP binding site of the enzyme (Druker *et al.* 1996; Druker and Lydon 2000). Imatinib specifically targets cells expressing *BCR/ABL* and does not affect normal cells (Druker *et al.* 1996; Deininger *et al.* 1997). This results in inhibition of downstream tyrosine phosphorylation by *BCR/ABL* (Druker and Lydon 2000). It is well tolerated in patients with CML and has significant anti-leukaemic activity (Druker *et al.* 2001). Treatment with imatinib results in a significant decrease in the levels of *BCR/ABL* transcripts (Muller *et al.* 2003).

Detection of *BCR/ABL* positive cells by real time PCR in patients undergoing treatment with imatinib is used to measure levels of residual disease during and after treatment. After three months of treatment this method can predict complete cytogenetic response within the first year (Muller *et al.* 2003). There has been some discussion about standardised procedures for monitoring *BCR/ABL* levels particularly with regard to an appropriate control gene. Initial guidelines suggested comparing expression of *BCR/ABL* to that of the untranslocated *ABL* allele (Gabert *et al.* 2003). However, using this approach primers designed to amplify normal *ABL* would also amplify *BCR/ABL* making interpretation of results problematic. It has now been suggested that more appropriate control genes should be considered such as  $\beta$ -glucuronidase or  $\beta 2M$  (Hughes *et al.* 2006).

The constitutive activation of the *BCR/ABL* fusion is known to result in activation of numerous signalling pathways. *BCR/ABL* promotes cell proliferation and survival by activating the RAS, SHP2 and PIK-AKT pathways and downregulating ICSBP and JUNB (Ren 2005). It also plays a role in regulating telomere maintenance pathways, for example, inhibition of *BCR/ABL* in cell lines expressing the fusion gene results in an increase in telomerase activation and tankyrase expression (Bakalova *et al.* 2004). *BCR/ABL* can stimulate reactive oxygen species that can cause DNA damage including mutations and double strand breaks (Nowicki *et al.* 2004). These are not repaired faithfully as *BCR/ABL* can promote unfaithful double strand break repair resulting in cytogenetic abnormalities and genomic instability which are thought to be required for progression of carcinogenesis (Skorski 2002; Nowicki *et al.* 2004).

Other proteins activated by *BCR/ABL* include RAS and JAK transducers and activators of transcription. *BCR/ABL* has been shown to phosphorylate the signal transducer and

activator of transcription STAT5 and this leads to constitutive activation of STAT5 (Ilaria and Van Etten 1996). STAT5 regulates cell proliferation, differentiation and apoptosis and its activation by BCR/ABL is thought to contribute to cell proliferation (Ilaria and Van Etten 1996; Nosaka *et al.* 1999). When STAT5 is activated by BCR/ABL it can induce expression of BCL-xL which has anti-apoptotic functions and may contribute to apoptosis resistance in CML cells (Horita *et al.* 2000). Additionally, BCR/ABL phosphorylates JAK2 to induce tyrosine kinase activation and this has been detected in peripheral blood of BC CML patients and may be a contributing factor in disease transformation in CML (Xie *et al.* 2001).

#### ***4.1.4 Aims of the study***

The aims of this study were to gain further insight into the mechanisms leading to the deregulated gene expression of telomerase associated genes identified in Chapter 3. The investigation of the methylation and mutation status of the *hTERT* promoter will further understanding of these processes in CML and investigate any involvement in levels of *hTERT* gene expression. Investigation into levels of the *BCR/ABL* fusion product will identify correlations with deregulated gene expression and disease progression.

## ***4.2 Materials and methods***

### ***4.2.1 Methylation specific PCR (MSP)***

Genomic DNA from patient and control samples as detailed in chapter 3 were bisulfite modified along with universally methylated and unmethylated DNA controls by means

of the CpGenome DNA modification kit (Chemicon/Millipore, Billerica, MA, USA). PCR was then carried out according the protocol in Chapter 2 using the primer sets detailed in Table 4.1 specific for methylated and unmethylated DNA in the 5' region and 3' region (including the transcription start site) of the *hTERT* promoter. Control primers were also included to identify any unmodified DNA. A touchdown PCR protocol was used where the annealing temperature is initially 70°C and decreases by 1°C each cycle for 10 cycles. A further 30 cycles were then performed with an annealing temperature of 60°C.

**Table 4.1 Methylation Specific PCR primers for *hTERT* promoter regions**

Primer Name	Sequence 5'-3'
hTERT 5U F	GTGGGTATAGATGTTTAGGATTGT
hTERT 5U R	CCACATACACAACAAAACACAACA
hTERT 5M F	GGGTATAGACGTTTAGGATCGC
hTERT 5M R	CGTACGCAACAAAACGCAACG
hTERT 3U F	AGTTTTGGTTTTGGTTATTTTTGT
hTERT 3U R	AAACACTAAACCACCAACACA
hTERT 3M F	AGTTTTGGTTTCGGTTATTTTCGC
hTERT 3M R	CAAACACTAAACCACCAACGCG
hTERT Nmod F	GGCCGATTCGACCTCTCT
hTERT Nmod R	AAGGTGAAGGGGCAGGAC

**4.2.2 Cycle sequencing of *hTERT* promoter regions**

The *hTERT* promoter regions were identified and primers were designed to amplify the appropriate regions using the primer 3 software. Primer sets 2 and 3 both include the known Ets2 site polymorphism. Genomic DNA from CD34<sup>+</sup> patient and control DNA samples was then amplified and subjected to cycle sequencing as detailed in Chapter 2. Primer sequences are detailed in Table 4.2 and annotated on the sequence in Fig 4.3



**Table 4.2 *hTERT* promoter PCR and sequencing primers**

Primer Name	Sequence 5'-3'
hTERT 1F	AACAGATTTGGGGTGGTTTG
hTERT 1R	GCCTAGGCTGTGGGGTAAC
hTERT 2F	CGTCCTCCCCTTCACGTC
hTERT 2R	CAGCGCTGCCTGAAACTC
hTERT 3F	GGCCGATTCGACCTCTCT
hTERT 3R	AGCACCTCGCGGTAGTGG

tccatttccc accctttctc gacgggaccg ccccggtggg tgattaacag atttggggtg  
gtttgctcat ggtggggacc cctcgccgcc tgagaacctg caaagagaaa tgacgggcct  
gtgtcaagga gcccaagtgc cggggaagtg ttgcaggag gcaactccggg aggtcccgcg  
tgcccgtcca gggagcaatg cgtcctcggg ttcgtcccca gccgcgtcta cgcgcctccg  
tcttccccctt cactctccgc attcgtggtg cccggagccc gacgccccgc gtccggacct  
ggaggcagcc ctgggtctcc ggatcaggcc agcggccaaa gggtcgccgc acgcacctgt  
tcccagggcc tccacatcat ggccccctcc tcgggttacc ccacagccta ggccgattcg  
acctctctcc gctggggccc tcgctggcgt ccctgcaccc tgggagcgcg agcggcgcg  
ggcggggaa gcgcggccca gacccccggg tccgcccga gcagctgcgc tgtcggggcc  
aggccgggct cccagtggat tcgcgggcac agacgccag gaccgcgctc cccacgtggc  
ggagggactg gggaccggg caccgtcct gcccttcac ctccagctc cgcctcctcc  
gcgcggaccc cgcgccgtcc cgacccctcc cgggtcccg gcccagcccc ctccgggccc  
tcccagcccc tccccttctt ttccgcggcc cgcgcccttc ctgcgggcgc gaggttcagg  
cagcgtgcg tctgtctgcg cactgtggaa gccctggccc cgccacccc cgcgatgccg  
cgcgtcccc gctgccgagc cgtgcgtcc ctgctgcgca gccactaccg ccagggtgctg

**Figure 4.3 *hTERT* promoter sequence.** The start codon is highlighted in red. The SNP at -245 bp is the C nucleotide highlighted in red. The E box sequences where c-Myc binds are highlighted in blue and Sp1 consensus motifs are highlighted in green. Ets2 binding sites are highlighted in grey and underlined. Primer sequences are underlined and detailed in Table 4.2.

**4.2.3 Measurement of *BCR/ABL* transcripts by real time PCR**

Multiplex PCR to identify *BCR/ABL* breakpoint was carried out on cDNA from all patient samples as detailed in chapter 2. Primer sequences were modified from those described by Cross *et al.* (Cross *et al.* 1994) to amplify *BCR/ABL* fusion transcripts as follows: Normal *BCR* 808bp, p190 *BCR/ABL* 481bp, p210 b3a2 *BCR/ABL* 385bp and p210 b2a2 *BCR/ABL* 310bp. The following primers were used in a multiplex PCR with an annealing temperature of 64°C:

C5e: 5'-CAGCTCTCCTTTGCAACCGGGTCTGAA-3'  
CA3: 5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3'  
BCR-C: 5'-ACCGCATGTTCCGGGACAAAAG-3'  
B2B: 5'-ACAGCATTCCGGTGACCATCAATAAG-3'

Absolute expression levels of *BCR/ABL* were calculated in relation to *β2M* against a plasmid standard curve of known cDNA copy number. For the *B2M* standard curve plasmid pLJCB2M.5 PCR products were generated from control cDNA using primers described in section 3.2.3 and cloned into the pGEM-T Easy vector system (Promega, Madison, WI). Positive clones were selected, extracted using Wizard plus minipreps (Promega) and then sequenced for confirmation of their insert using M13 sequencing primers (Big Dye Terminator cycle Sequencing Kit, Version 1.1, and ABI 3100 Genomic Analyser, Applied Biosystems). For quantification of p210 *BCR/ABL* the plasmid pNC210/G was used, kindly provided by Dr J Kaeda (Hammersmith Hospital, London, UK).

Plasmids were quantified spectrophotometrically and the mass of a single plasmid was calculated from the size of the vector and insert. This value was used to calculate the concentration of plasmid DNA needed to achieve the copy numbers of interest. Plasmids were linearised and serially diluted in nuclease free H<sub>2</sub>O with 50µg/ml *E.coli* 16s and 23s rRNA (Roche, Mannheim Germany) as a background nucleic acid to mimic the unknown samples. Successive dilutions were prepared in the range  $10^7$  -  $10^3$  copies for pNC210/G and  $10^9$  -  $10^4$  for pLJCB2M.5, with steps at every half order of magnitude on a logarithmic scale. Correlation coefficients ( $r^2$ ) for calibration curves were always greater than 0.99 and mean slopes of the curves were -3.47 for B2M and -3.31 for p210.

The standard curve method was used to extrapolate the exact copy number present in the patient samples. A standard curve of plasmid DNA calibrators was derived by plotting the Ct values for each plasmid dilution against the log copy number of the plasmid. *B2M* was used as the control gene to compensate for any differences in input RNA and efficiency of the reverse-transcriptase reaction. *BCR/ABL* primers and probe were as described by Gabert (Gabert *et al.* 2003). Normalised copy number of *BCR/ABL* transcripts was expressed as a percentage of *B2M* transcript copy number.

### **4.3 Results**

#### **4.3.1 *hTERT* promoter methylation status in CML**

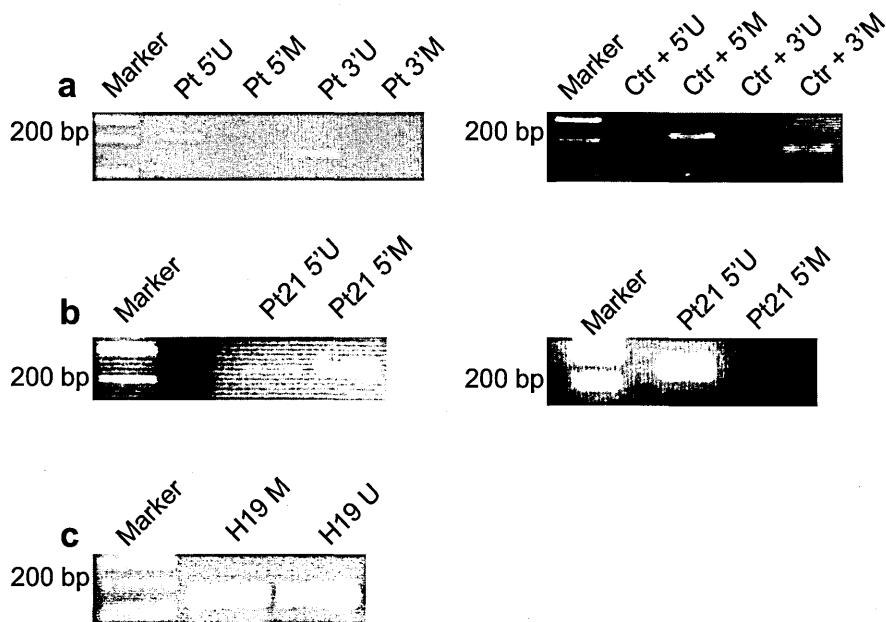
The *hTERT* promoter region CpG island was identified from the genbank file AF097365 corresponding to the *hTERT* promoter region and partial coding sequence using MethPrimer online software (Li and Dahiya 2002). Genomic DNA from CD34<sup>+</sup> cells of CML patients and normal controls was first subjected to bisulfite treatment, which will convert all unmethylated cytosines to uracil. PCR using primers specific for methylated or unmethylated DNA was then used to distinguish the methylation status of the DNA. Two promoter regions of *hTERT* representative of the 5' and 3' ends of the *hTERT* CpG island were amplified using primer pairs specific for methylated or unmethylated DNA. All patients and controls described in Chapter 3 were used for this part of the study. Amplified DNA fragments were visualized by means of agarose gel electrophoresis.

#### ***4.3.1.1 hTERT promoter methylation in CD34<sup>+</sup> cells***

Methylation specific PCR (MSP) shows that both the 3' and 5' promoter regions of the *hTERT* promoter are not methylated in the CD34<sup>+</sup> cells of CML patients and of normal controls. In Fig 4.4a it can be seen that only the primers specific for unmethylated DNA yield a PCR product in these samples. There was, however, one exception: The 5' promoter region of the sample from patient 21 taken in accelerated phase of disease. A PCR product was produced using primers specific for both methylated and unmethylated DNA in this region. However, this result was not reproducible (Fig 4.4b). Two separate PCR amplifications give product for methylated and unmethylated DNA and two additional separate PCR amplifications give product for unmethylated DNA only. Methylated DNA in the 3' promoter region of this sample was not amplified by the primers specific for methylation in this region.

To further investigate this result the methylation specific PCR products were sequenced in a selection of cases including pt 21. Cycle sequencing was carried out using the ABI 3100 machine with the MSP primers. Analysis of the sequencing data shows that all CpG sites amplified by the primers specific for unmethylated DNA are not methylated. Sequencing of PCR products specific for methylated DNA shows that 12 out of 13 CpG sites in this region are methylated in patient 21. Fig 4.5

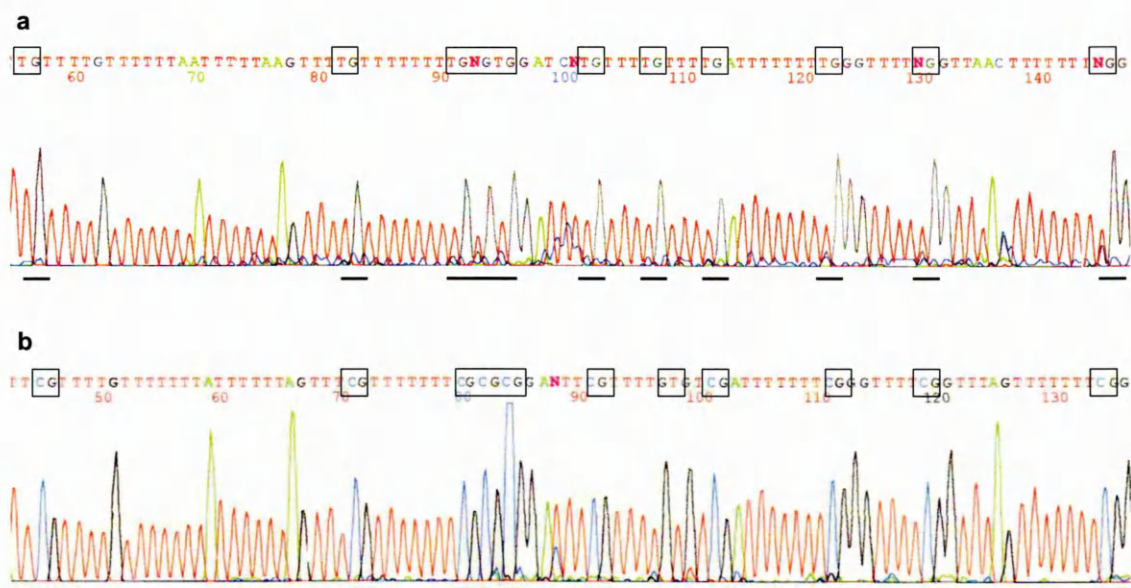
Since all but the sample from pt 21 do not yield any PCR product using primers specific for methylated DNA it is important to include a positive control for these primers. Universally methylated DNA was used as a positive control for methylated primers to confirm successful PCR amplification, as shown in Fig 4.4a.



**Figure 4.4 Methylation Specific PCR of the *hTERT* promoter region.** U= PCR with primers specific for unmethylated DNA, M= PCR with primers specific for methylated DNA. (a) MSP of CD34<sup>+</sup> cell DNA from one patient sample and from universally methylated (+) DNA controls. (b) MSP of DNA from patient 21 showing amplification of methylated and unmethylated DNA in the left hand panel and only unmethylated DNA in the right hand panel. (c) MSP of *H19* in a patient sample showing amplification of methylated and unmethylated DNA.

#### 4.3.1.2 *hTERT* promoter methylation in Neutrophils

To investigate methylation between different cell types and disease bisulfite modified DNA from peripheral blood neutrophils of 10 CML patients, four cases of Acute Myeloid Leukaemia and 6 normal controls was tested. This DNA was amplified using the primers specific for methylated or unmethylated *hTERT* promoter DNA. This Methylation specific PCR shows that the *hTERT* promoter is not methylated in the 5' and 3' CpG sites in neutrophils of CML and AML patients or in normal control neutrophils. A neutrophil DNA sample from pt 21 in AP was included in this analysis and this was also negative for methylation of both *hTERT* promoter regions.



**Figure 4.5 Cycle sequencing of Methylation specific PCR products.** CpG islands are highlighted by black boxes and underlined. (a) Unmethylated C has been converted to T by bisulfite modification. (b) Sample from pt 21. Methylated C has been protected from bisulfite modification by methylation.

#### 4.3.1.3 Specificity of Methylation Specific PCR

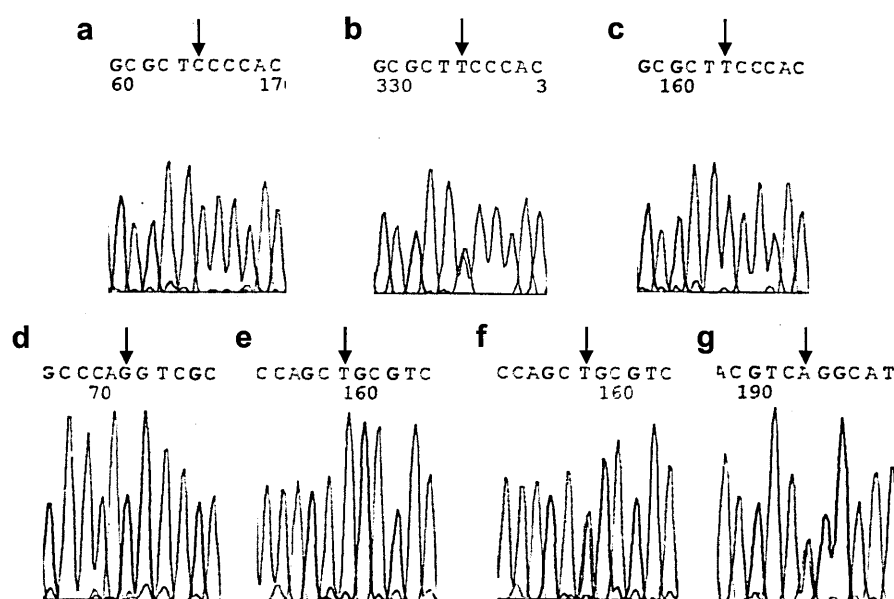
To ensure that any results obtained from methylation specific PCR of patient samples were that from genuine DNA of defined methylation status a control gene of known methylation status was amplified for all patient and control samples. The imprinted *H19* gene gives a positive control for the detection of methylation status in these cells, one copy is always methylated and one copy is always unmethylated. Therefore, PCR specific for methylated or unmethylated *H19* DNA should always be positive in patient and control samples. This was the case for all samples analysed, see Fig 4.4c.

#### 4.3.2 *hTERT* promoter mutation status

Cycle sequencing of the *hTERT* promoter using three overlapping primer sets was used to analyse the *hTERT* promoter in genomic DNA from the CD34<sup>+</sup> cells of CML patients and normal controls. DNA was not available for three of the patients. Differences between the known *hTERT* promoter sequence and sequences obtained from the samples studied were identified using pairwise analysis of sequences using Sequence Analysis software.

Mutation analysis of the *hTERT* promoter has confirmed the presence of a SNP at -245 bp from the *hTERT* transcription start codon in the Ets2 binding site, as reported by (Hsu *et al.* 2006) (Fig 4.6a,b,c). This SNP was found to have the genotype C/C, T/C or T/T. The most common genotype in patient and control groups was T/T, 12 patients and 8 of 11 normal controls had this genotype. Five patients and one control had the C/C genotype and four patients and two controls were T/C heterozygotes. Where serial DNA samples were available from a single patient (Pt 17, Pt 21, Pt 22) the *hTERT* promoter SNP genotype did not change between samples. It has been previously reported that the C/C and T/C genotypes correlated with lower telomerase activity (Hsu *et al.* 2006). The genotype of the SNP was correlated with *hTERT* expression in patients and controls but there was no significant difference in the levels of *hTERT* expression between the samples with T/T genotype versus the C/C and C/T groups. Similarly there was no difference in expression observed when comparing the C/C genotype to the C/T genotype. Therefore, no correlation was identified between T/T, T/C or C/C genotype at the Ets2 binding site and reduced expression of *hTERT*. In fact, one of the samples with highest *hTERT* expression levels (ratio 1.64) was Pt11 who was homozygous for the C/C genotype.

Additional base changes were found in three patients and two normal controls. One patient and one control had a heterozygous C to T mutation at position -613 (Fig 4.6f). A further control sample had a homozygous C to T change of this nucleotide (Fig 4.6e). This control also had a homozygous A to G mutation at position -699 (Fig 4.6d). These mutations did not correlate with *hTERT* expression. Two further patient samples showed a heterozygous C to A base change at position -578 (Fig 4.6g). This mutation was not seen in any of the normal control samples. Comparing *hTERT* expression in these two patients pt14 and pt20 ratio 0.08 and 0.95 respectively it appears that this change does not correlate with *hTERT* expression.



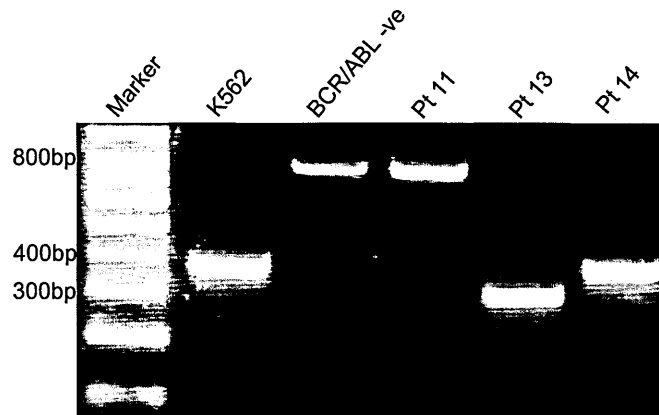
**Figure 4.6 *hTERT* promoter sequence variation in CML.** Variation is indicated by a black arrow. (a) C/C genotype at -245 bp. (b) T/C genotype at -245 bp. (c) T/T genotype at -245 bp. (d) A to G substitution at -699 bp. (e) C to T substitution at -613 bp. (f) A heterozygous C to T substitution at -613 bp. (g) A heterozygous C to A substitution at -578 bp.



### 4.3.3 *BCR/ABL* transcript levels in CML

#### 4.3.3.1 *Identification of transcript variant in patient samples*

The Philadelphia chromosome has two common breakpoints. *BCR* exon b2 or b3 is fused to *ABL* exon a2 giving rise to the p210 fusion protein seen in the majority of CML patients. In sporadic cases of CML *BCR* exon e1 is fused to *ABL* exon a2, encoding the p190 fusion protein. It was therefore necessary to establish the transcript variant that each patient expressed in order to use the appropriate real time PCR assay. cDNA of CML patients was typed for transcript variant using multiplex PCR primers designed to produce PCR products of different sizes corresponding to the three possible breakpoints in *BCR*. This method uses four primers designed to give different bands depending on the fusion transcript: Normal *BCR* 808bp, p190 *BCR/ABL* 481bp, p210 b3a2 *BCR/ABL* 385bp and p210 b2a2 *BCR/ABL* 310bp. Visualisation of these PCR products by agarose gel electrophoresis reveals that all patients studied express the most common p210 *BCR/ABL* transcript variant, Fig 4.7. In patients treated with imatinib only the normal *BCR* gene and not the *BCR/ABL* fusion transcript was detected by PCR. Only one type of fusion transcript was detected in each patient. The cell line K562 was included as a positive control for *BCR/ABL* amplification and normal control neutrophils as a control for normal *BCR* amplification.



**Figure 4.7 Agarose gel electrophoresis of *BCR/ABL* transcript variant PCR.** PCR products were resolved on a 1.8% agarose gel. K562: cDNA from the K562 cell line, *BCR/ABL* -ve: normal control neutrophil cDNA. Pt11 is normal *BCR*, Pt13 is P210 b2a2 and pt14 is P210 b3a2.

#### 4.3.3.2 Real time PCR monitoring of *BCR/ABL* transcript levels

Absolute expression levels of *BCR/ABL* were calculated in relation to  $\beta 2M$  against a plasmid standard curve of known cDNA copy number. Levels of *BCR/ABL* transcripts are expressed as a percentage of  $\beta 2M$  transcripts. Statistically significant differences in expression levels between the patient groups were investigated using Mann-Whitney or Kruskal-Wallis non parametric tests where appropriate. Results are summarised in table 4.3. Expression levels of *BCR/ABL* in chronic phase were variable ranging from 0.07 to 109.6 with a median of 23.5. Excluding samples from patients treated with imatinib, three out of the twelve samples from chronic phase were receiving treatment. These three patients had *BCR/ABL* expression levels below the median for CP and pt1, who had been in CP for twelve years and was being treated with interferon, had the lowest expression level in this group at 0.07. Median *BCR/ABL* expression for untreated CP patients is 27.7 and for the three patients treated with interferon or hydroxyurea median *BCR/ABL* is 0.17. These differences are not quite significant ( $p=0.063$ ). *BCR/ABL*

Levels in AP and BC patients appear more constant than the CP group and median expression is slightly lower than CP at 20.3 and 20.93, respectively. These differences do not reach statistical significance ( $p=0.19$ ). All patients in AP and BC were receiving some kind of treatment, see table 3.1. In a single patient (pt21) where serial samples are available *BCR/ABL* levels decrease with disease progression from 39.09 at diagnosis in CP to 20.27 in AP and 20.93 in BC following treatment with hydroxyurea.

**Table 4.3 *BCR/ABL* expression levels in CML patients.**

Disease Status	Range	Median
CP	0.07 – 109.6	23.5
AP	12.8 – 25.2	20.3
BC	10.4 - 51.9	20.93
STI	$5.6 \times 10^{-5}$ - 0.38	0.002

**4.3.3.3 Real time PCR for *BCR/ABL* transcript levels in patients treated with imatinib**

Samples were available from seven patients following three months of treatment with imatinib. The action of this drug reduces the number of *BCR/ABL* positive cells and therefore reduces *BCR/ABL* transcript levels. All patients showed partial or complete cytogenetic response to treatment after three months. Levels of *BCR/ABL* in patients treated with imatinib were very low with expression levels ranging from  $5.6 \times 10^{-5}$  to 0.38 and a median of 0.002. Moreover, expression was significantly reduced as compared to CP patients,  $P=.0008$ . Median expression of *BCR/ABL* in the patients treated with imatinib is lower than the CP patients undergoing other treatments, however these differences are not statistically significant. In serial samples from two patients treated with imatinib *BCR/ABL* levels were greatly reduced after three months

of treatment. In pt 17 *BCR/ABL* levels decrease from 109.6 in untreated CP to 0.38 after treatment and in pt 22 levels decrease from 27.7 to 0.001 after treatment.

#### **4.4 Discussion**

Investigation of the methylation status of the *hTERT* promoter region in both CD34<sup>+</sup> cells and neutrophils reveals that the promoter region is not methylated in CML or normal control samples. In general, methylation of the promoter region of a gene results in reduced expression of that gene and there is evidence to suggest that this is the case for *hTERT*. Investigation of methylation in the bone marrow of patients with B-cell lymphocytic leukaemia reveals that those patients that have low telomerase activity also exhibit more *hTERT* promoter methylation than those patients with high telomerase activity (Bechter *et al.* 2002). In contrast to this it has been demonstrated that HL-60 leukaemia cells treated with a demethylating agent exhibit reduced *hTERT* expression (Hajek *et al.* 2008). It has been shown in cancer cells lines that *hTERT* is expressed despite promoter methylation (Guilleret *et al.* 2002). It has been suggested high levels of promoter methylation of *hTERT* can be present as long as levels of methylation around the transcription start site are low or absent (Zinn *et al.* 2007). In the current study this region was amplified by the primers specific for the 3' region of the promoter and no methylation was found. Absence of promoter methylation of *hTERT* can decrease transcription by allowing the binding of negative regulators of transcription. For example, the ubiquitously expressed CCCTC-binding factor (CTCF) which binds to GC-rich sequences to inhibit transcription in the absence of DNA methylation (Lobanenko *et al.* 1990; Renaud *et al.* 2007). CTCF has been shown to inhibit *hTERT* transcription in this way (Renaud *et al.* 2005). CTCF binds the first exon of the *hTERT* gene, the majority of which was amplified by the primers used in this study to amplify

the 3' region of the *hTERT* CpG island (Renaud *et al.* 2007 122). This region was shown to be unmethylated in patient and control samples. It can be speculated that the absence of methylation around this site and the other promoter regions studied is not directly effecting the expression of *hTERT* in the CML patients. Therefore, methylation of the *hTERT* promoter region does not appear to cause the reduction in *hTERT* expression in patients with CML. However the absence of methylation in these cells could lead to repression of *hTERT* transcription by allowing the binding of CTCF which would lead to the observed reduction in transcription. This reveals another potential mechanism underlying the reduced *hTERT* expression seen in the CML patients and it would be of interest to investigate if aberrant expression of *CTCF* is involved in disease progression of CML.

The one exception to this is a sample from pt 21 in AP where a positive result for methylation was seen using primers amplifying the 5' promoter region. However, this was not reproducible. It is unlikely that this result was due to contamination as there was never product seen in the negative control and the result was restricted to this sample. It may be the case that a small number of clones were present in this sample which did have methylation of the *hTERT* promoter. However, as there are serial samples available from this patient which were all negative for methylation it may be the case that this result was an artefact. Additionally, methylation in this region will not necessarily have an effect on *hTERT* expression as it has been demonstrated that the *hTERT* promoter can be methylated and still express *hTERT* as long as the region around the start site (amplified by the 3' primers) is not methylated (Zinn *et al.* 2007).

Sequencing of the *hTERT* promoter region was carried out to investigate the presence of a SNP in the Ets2 binding site at -245 bp from the start codon that results in decreased expression of *hTERT* (Hsu *et al.* 2006). The region sequenced also contains two E-

boxes where c-Myc will bind and an SP-1 consensus motif where the general transcription factor SP-1 binds. In agreement with the study by Hsu *et al.* the most frequent genotype detected for the SNP at -245 was T/T homozygote. In the samples with either C/T or C/C genotype it was not possible to confirm the relationship between this genotype and reduced *hTERT* expression. This suggests that in the CD34<sup>+</sup> cells of the CML patients in the current study the SNP at -245 bp is not regulating *hTERT* expression. A total of twelve samples were shown to have either C/T or C/C whereas in the study by Hsu *et al.* 57 samples had either of these two genotypes. It may be the case that a higher sample number in the present study would increase the statistical power allowing a relationship to be observed.

ETS2 has been shown to directly interact with c-MYC in breast cancer cell lines (Xu *et al.* 2008). When c-MYC is reduced ETS2 binding at its site in the *hTERT* promoter is inhibited and the authors suggest that these two proteins form a complex when binding the promoter and together regulate *hTERT* transcription. Additionally, *ETS2* expression is required for expression of *c-MYC* and in the absence of these two proteins *hTERT* transcription is switched off. In the present study *c-MYC* expression is reduced and this may lead to reduced binding of ETS2 at the *hTERT* promoter which could contribute to the reduced *hTERT* expression. It can also be suggested that ETS2 may itself be reduced and this could be a cause of a reduction in c-Myc expression. However, reports of gene expression analysis in CML suggest that *ETS2* expression is increased in CML (Nowicki *et al.* 2003).

The other mutations observed in the *hTERT* promoter region were not in the region of known binding sites of transcriptional activators or repressors of *hTERT*. The base changes seen here may be infrequent SNPs that do not influence transcription levels of *hTERT*. Therefore, it could be concluded that the base pair changes observed in the

*hTERT* promoter are not a direct cause of the reduced *hTERT* expression observed in CML.

Expression levels of *BCR/ABL* were measured in the CD34<sup>+</sup> cells of CML patients by real time PCR. Analysis of patients in CP that were sampled at diagnosis and before treatment had higher *BCR/ABL* levels than the patients who were undergoing treatment. This includes patients that were treated with interferon (Pt 1 and Pt 19) or hydroxyurea (Pt 13). Treatment with these agents can control CML and patients do have a cytogenetic response, although this is rare with hydroxyurea. This suggests that a decrease in the numbers of *BCR/ABL* positive cells would be expected after treatment. Therefore the reduced expression in the three patients treated with interferon or hydroxyurea could be a reflection of the choice of treatment. In fact, in a CML cell line KT-1, treatment with interferon reduced the expression of the *BCR/ABL* fusion gene (Yanagisawa *et al.* 1998). Additionally, using real time PCR monitoring it has been shown that *BCR/ABL* transcript levels are reduced in patients after treatment with interferon and some patients achieve a complete cytogenetic response (Muller *et al.* 2003). This is likely to be the case for Pt 1, who had been in CP for twelve years and was being treated with interferon and had the lowest *BCR/ABL* expression level in this group at 0.07. This suggests that the number of leukaemic CD34<sup>+</sup> cells in this patient have been greatly reduced by treatment.

Serial samples were available for pt 21 in disease progression from CP, AP and BC. It may be expected that as the disease is progressing expression of *BCR/ABL* would increase. However, this was not the case and expression of *BCR/ABL* in AP and BC was lower than the CP sample at diagnosis from this patient. This patient was being treated with hydroxyurea in AP and BC which inhibits ribonucleotide reductase to stop

cells dividing and is thought to increase survival in CML patients by slowing proliferation rates of granulocytes (Hehlmann *et al.* 1993). This could mean that CD34<sup>+</sup> cell proliferation rates in this patient may be regulated in part by treatment with hydroxyurea resulting in steady levels of *BCR/ABL* transcripts after treatment.

In the patients treated with imatinib *BCR/ABL* transcript levels are greatly reduced compared to untreated CP patients. Imatinib directly inhibits the *BCR/ABL* fusion protein to reduce the number of leukaemic cells. This results in a decrease in levels of *BCR/ABL* transcripts as seen in the current study. This result is in agreement with several other studies that also show reduced *BCR/ABL* expression after treatment with imatinib (Wang *et al.* 2002; Kantarjian *et al.* 2003; Muller *et al.* 2003; Press *et al.* 2006). This reflects the return of normal haematopoiesis as cells expressing *BCR/ABL* are eliminated. As described in chapter 3 it has been shown in the current study that levels of *hTERT* transcription in the patients treated with imatinib are increased when compared with untreated CP patients. In the treated patients a decrease in *BCR/ABL* expression correlates with an increase in *hTERT* expression. It could be suggested that this decrease in *BCR/ABL* expression could lead to the increase in *hTERT* expression as the cells expressing *BCR/ABL* are eliminated. This is in agreement with a study showing that inhibition of *BCR/ABL* in human cell lines promotes *hTERT* expression and this appears to be happening in the patients in this study that were treated with imatinib (Bakalova *et al.* 2004). Interestingly, it has been shown that in patients that have undergone long term treatment with imatinib telomeres are extended (Brummendorf *et al.* 2003). This suggests a link between the decrease in *BCR/ABL* after treatment with imatinib and increased *hTERT* expression leading to an increase in telomere length as the leukaemic cells are eliminated.



The reduction of *hTERT* expression is observed in the CD34<sup>+</sup> cells of CML patients may be partly caused by the BCR/ABL fusion protein inhibiting *hTERT* expression. This effect could be enhanced by the absence of methylation of the *hTERT* promoter which would allow binding of negative regulators of transcription such as CTCF. Mutation of promoter regions of *hTERT* do not appear to play a role in reducing expression, however, a reduction in *c-MYC* expression could reduce binding of the positive transcriptional activator ETS2 to its promoter binding site contributing to the reduction of *hTERT* expression.

***Chapter 5***

***Gene Expression Profiling of***

***Platelets and Neutrophils of Patients***

***with Essential Thrombocythaemia***

## 5.1 Introduction

### 5.1.1 Clinical characteristics of ET

The myeloproliferative neoplasms are clonal disorders resulting in an overexpansion of cells of the myeloid lineage and include Chronic Myeloid Leukaemia (CML), Essential Thrombocythaemia (ET) and Polycythaemia Vera (PV). PV typically has increased erythrocytosis but it is common for patients to have proliferation of the erythroid, megakaryocyte and granulocyte lineages (Levine and Gilliland 2008). Where CML exhibits an over proliferation of granulocytes ET displays increased megakaryocyte proliferation (Tefferi and Vardiman 2008). ET is characterised by dysmorphic megakaryocyte production leading to excessive platelet production resulting in a significant risk of thrombohaemorrhagic complications. (Wolanskyj *et al.* 2005) However, the median survival of patients is more than 20 years and up to 10% will transform into an acute leukaemia. (Wolanskyj *et al.* 2005) Karyotypic abnormalities in ET are rare and it is estimated that only 5% of patients have cytogenetic abnormalities at diagnosis and these do not appear to be prognostically useful (Beer *et al.* 2011). Trisomy 8 and 9 have been observed in multiple cases of ET pre transformation (Elis *et al.* 1996). Duplication of the long arm of chromosome 1, deletion of 20q and 13q are also observed in numerous cases (Bench *et al.* 2001; Steensma and Tefferi 2002). Approximately 3% of patients carry the mutation *MPL* W515L/K which does not appear to represent a distinct disease phenotype but can be of diagnostic importance (Vannucchi *et al.* 2008). The most common molecular abnormality in these patients is the *JAK2*V617F mutation (Beer *et al.* 2011).

### 5.1.2 The *JAK2 V617F* mutation

JAK2 is a member of the Janus kinase family and is a ubiquitously expressed protein tyrosine kinase that functions to mediate cytokine signalling. (Kisseleva *et al.* 2002) Cytokines bind haematopoietic cytokine receptors and use JAK kinases to initiate signal transduction which regulates the proliferation and differentiation of haematopoietic cells. (Murray 2007)

The *JAK2 V617F* mutation was first described in 2005 by several groups and was reported to be present in the majority (approximately 95%) of patients with PV and approximately 50-60% of ET patients (Baxter *et al.* 2005; James *et al.* 2005; Kralovics *et al.* 2005; Levine *et al.* 2005; Zhao *et al.* 2005). The mutation, in exon 14 of the *JAK2* gene on chromosome 9p24 is a G to T point mutation that results in valine at amino acid 617 being substituted with phenylalanine. In those patients where the mutation was homozygous it has been shown that this homozygosity was a result of mitotic recombination (Baxter *et al.* 2005; Kralovics *et al.* 2005).

The mutation affects the pseudokinase domain of JAK2 which is thought to have a function in inhibiting kinase domain activity (Saharinen *et al.* 2000). The mutation results in a conformational change of the pseudokinase domain leading to constitutive activation of the kinase activity (Zhao *et al.* 2005). Similar to the activated kinase BCR/ABL being sufficient to cause CML, *JAK2 V617F* has been shown to be sufficient for development of PV in mice receiving a bone marrow transplant which was transduced with *JAK2 V617F* (Lacout *et al.* 2006; Wernig *et al.* 2006).

Several groups have investigated the differences between *JAK2 V617F* positive and negative patients with ET and PV. These studies are in agreement that ET patients with

the mutation have higher haemoglobin concentrations, neutrophil counts and total white cell counts than those without (Antonioli *et al.* 2005; Campbell *et al.* 2005; Wolanskyj *et al.* 2005; Zhang *et al.* 2008). These characteristics are phenotypically similar to PV and it has been suggested that the mutation divides ET patients into two subtypes: One type *JAK2* V617F positive with similar characteristics to PV; and the other *JAK2* V617F negative exhibiting classical clinical features of ET (Campbell *et al.* 2005). Although the *JAK2* mutation is diagnostically important it cannot be used to distinguish between ET and PV, therefore histological analysis is still essential for diagnosis of these patient samples. The factors responsible for ET and its transformation to acute leukaemia remain largely unknown.

### **5.1.3 Platelets and markers**

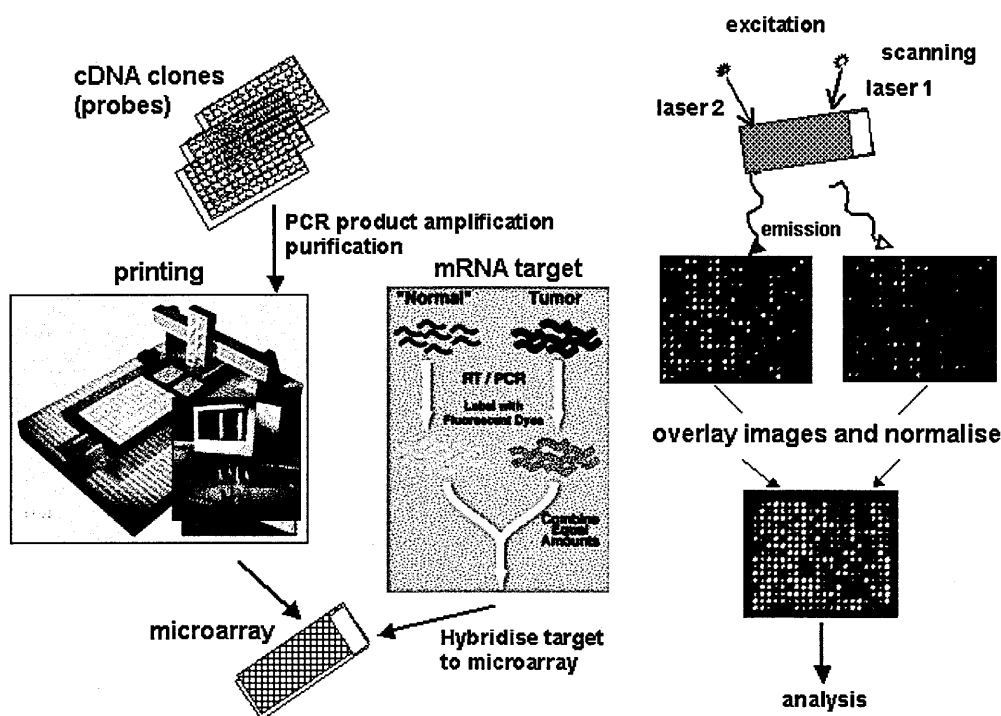
Platelets are cytoplasmic fragments produced from megakaryocytes and contain no genomic DNA (Newman *et al.* 1988). However, they do retain some RNA (estimated to be 0.002 fg per cell) derived from megakaryocytes and the translational machinery required for protein production (Newman *et al.* 1988; Schedel and Rolf 2009). Platelets are activated by the coagulation cascade and have a role in maintaining vascular integrity (Ho-Tin-Noe *et al.* 2011).

There have been limited studies characterising the RNA from platelets by microarray. These studies have shown that the most abundant transcripts in platelets are those encoding well characterised platelet genes such as Platelet factor 4 (*PF4*), glycoprotein IIb and glycoprotein Iba (*GP1BA*). Genes involved in cytoskeletal organisation and immune response were also present. Interestingly, several genes with unknown function in platelets were also expressed: *neurogranin* which is a PKC substrate and

*Clusterin* which inhibits complement lysis. Given the limited amount of RNA in platelet samples it is important to achieve high purity of the sample before analysis.

#### **5.1.4 cDNA Microarray analysis**

DNA microarrays are an important tool for the analysis of relative abundance of mRNA transcripts in a cell population. The arrays allow the analysis of global gene expression in many different scenarios such as disease versus normal, changes in expression during the cell cycle or development (Gershon 2002). cDNA arrays consist of cDNA or oligonucleotide sequences that are spotted onto a glass microscope slide by high precision robots (Holloway *et al.* 2002). The cDNAs are typically PCR products from cDNA clone sets such as the IMAGE consortium clones or oligonucleotides ranging in size from 50 -70 bases that are specific to known genes (Holloway *et al.* 2002). To compare the gene expression patterns in two different samples by cDNA microarray analysis the samples are first labelled with either Cy3 or Cy5 fluorescent dyes. The labelled samples are then competitively hybridised to the same microarray slide where the labelled nucleic acids will hybridise specifically to their corresponding cDNA on the array. The fluorescence intensity of each dye on each spot on the microarray is then measured by a specialised scanner and the measurements are overlaid to provide a gene expression ratio for each gene. The experimental process is summarised in Fig 5.1. The arrays used in this study were provided by the Sanger Institute (Cambridge, UK) and contain 9,932 cDNA spots representative of 6,000 known human genes and ESTs which can all be analysed simultaneously.



**Figure 5.1 Overview of a cDNA microarray experiment.** Image adapted from Duggan *et al.* 1999.

### 5.1.5 Aims of the study

The aims of this section of the study are to investigate the feasibility of separating a pure population of platelets from peripheral blood and to analyse these samples by gene expression profiling. Neutrophil samples will also be collected to investigate differences in gene expression of *JAK2* V617F positive neutrophils in ET and PV. It is hoped that microarray analysis will identify differentially expressed genes to aid with diagnosis of these disorders where currently the only widely used molecular marker is the *JAK2* V617F mutation. These samples will also be compared with neutrophils from CML patients expressing the activated tyrosine kinase of the *BCR/ABL* fusion transcript which is the definitive molecular marker for CML.

**5.2 Materials and Methods**

**5.2.1 Samples**

Neutrophil fractions were separated using the Histopaque reagent from peripheral blood of twelve ET, three PV and five normal controls as described in chapter 2. Patient details are shown in table 5.1. RNA and genomic DNA were extracted from these samples using TRIzol reagent. A normal pool of healthy control RNA was created by mixing equal amounts of RNA from each of the normal control samples.

**Table 5.1 Details of ET and PV patients.**

<b>Patient Identifier</b>	<b>Disease Status</b>
<b>M1352</b>	ET
<b>M1353</b>	ET
<b>M1354</b>	ET
<b>M1357</b>	ET
<b>M1358</b>	PV
<b>M1360</b>	PV
<b>M1372</b>	ET
<b>M1377</b>	ET
<b>M1378</b>	ET
<b>M1379</b>	ET
<b>M1381</b>	PV
<b>M1391</b>	ET
<b>M1395</b>	ET
<b>M1415</b>	ET
<b>M1421</b>	ET

**5.2.2 Separation of platelets from peripheral blood**

Platelets were separated from peripheral blood of patient and control samples by centrifugation and removal of platelet rich plasma as detailed in Chapter 2. Levels of contaminating cells were assessed by flow cytometry by labelling aliquots of



fractionated cells with FITC-Conjugated anti-CD41 to detect platelets, PE-conjugated anti-glycophorin A to label red blood cells and PerCP-conjugated anti-CD45 to label all leukocytes. Levels of individual cell types were analysed by flow cytometry using the FACSCalibur flow cytometer (BD Biosciences, Oxford, UK).

### **5.2.3 Cycle sequencing of JAK2**

Cycle sequencing was carried out as described in Chapter 2. Primers to amplify *JAK2* were used as described by James *et al.* (James *et al.* 2005). Forward Primer 5'-GGGTTTCCTCAGAACGTTGA-3' and Reverse Primer 5'-TTGCTTTCCTTTTTCACAAGA-3'. The primers used for PCR were subsequently used to sequence the resulting PCR product.

### **5.2.4 cDNA microarray analysis**

For every cDNA microarray experiment, neutrophil RNA from one patient or control sample was compared to the normal RNA pool. The Amino Allyl MessageAmp aRNA kit (Ambion) was used to for cDNA synthesis, antisense RNA (aRNA) synthesis and amplification and dye incorporation. For each patient and control pool sample 2 µg of total RNA was used. The normal control pool was labelled with Cy3 NHS ester dye and each patient sample was labelled with Cy5 NHS ester dye. The two labelled samples were then mixed and competitively hybridised to the cDNA microarray. Hybridisation was carried out at 47°C overnight and slides were washed. All experiments were performed in duplicate.

Slides were scanned using the ScanArray 4000 machine (PerkinElmer) with a 10- $\mu$ m resolution. This generated TIFF images which were imported in QuantArray 3.0 (PE/Packard BioScience). Grid alignment and spot location were carried out and the Cy5 and Cy3 intensities were normalised to the median value of the entire array and the ratio for every spot was obtained. Analysis files were imported into GeneSpring 7.2 (Silicon Genetics, Redwood City, CA) and gene filtering and clustering performed.

### **5.2.5 Real Time PCR**

RNA was extracted from patient and control neutrophil samples with TRIZOL reagent as described in chapter 2. RNA was then DNase treated using the DNA-free reagent (Ambion) and cDNA synthesis was carried out as detailed in chapter 2 using the Ambion RETROscript kit.

To account for differences in input cDNA Beta-2-microglobulin ( $\beta 2M$ ) expression was examined with a pre-developed assay (Applied Biosystems). The primer and probe sets used for the genes of interest were supplied from the assays on demand resource from Applied Biosystems. The assay numbers are as follows:

SNCA: Hs00240906

EGR3: Hs00231780

CCR5: Hs00152917

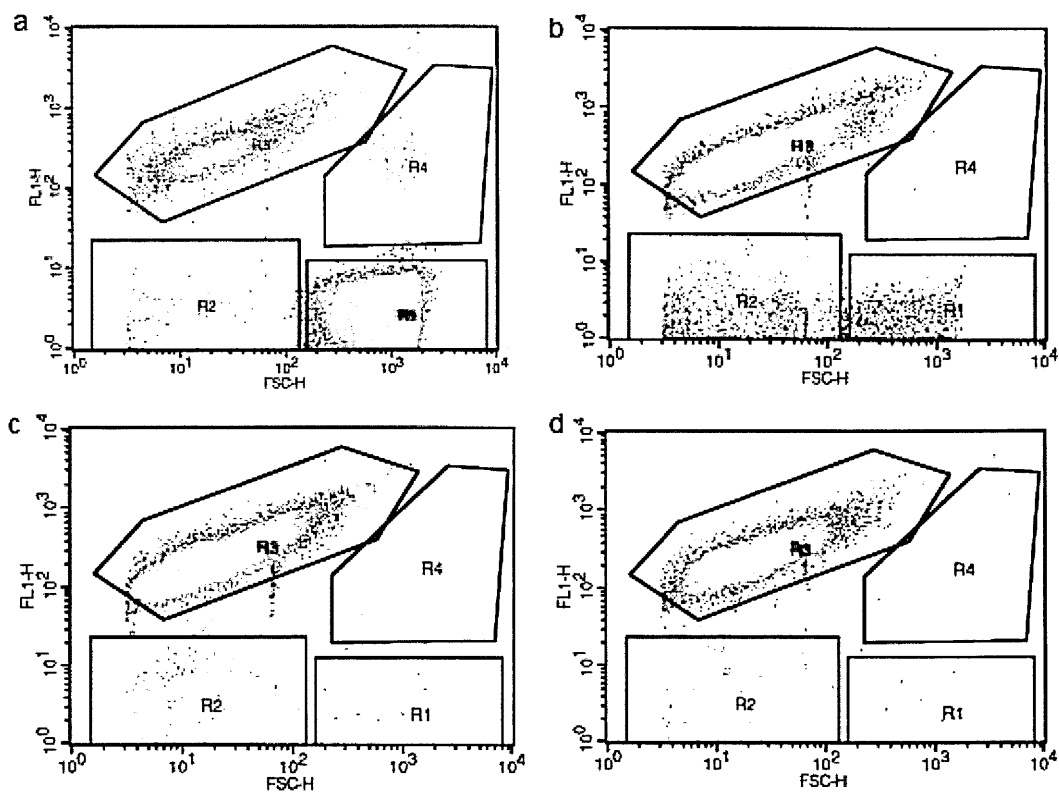
FCER1G: Hs00175408

Reactions were set up to run on the ABI prism 5700 Sequence Detection system and expression ratios were obtained using the  $2^{-\Delta\Delta ct}$  method.

## **5.3 Results**

### **5.3.1 Platelet separation from whole peripheral blood**

Using a method described by Wang *et al.* (Wang *et al.* 2003) 55 ml of whole peripheral blood was used to determine if a highly pure population of platelets could be isolated from a normal control. At each step of the protocol aliquots of the sample were labelled with fluorescent antibodies for detection of cell types by flow cytometry: FITC-Conjugated anti-CD41 will detect platelets, PE-conjugated anti-glycophorin A will label red blood cells and PerCP-conjugated anti-CD45 will label all leukocytes. Flow cytometry of whole blood shows that all cells are labelled as expected and clearly visible, see fig 5.2a. After spinning the blood at 150g and removing the Platelet Rich Plasma (PRP) a decrease in contaminating leukocytes and erythrocytes is seen, as shown in Fig 5.2b. A further centrifugation at the same speed shows an increase in the platelet concentration and a notable reduction in red blood cells (Fig 5.2c). A final centrifugation step at 880g yields a purified platelet pellet with minimal detection of other contaminating cell types (Fig 5.2d).



**Figure 5.2 Platelet Purification from Peripheral Blood.** Flow cytometry data: Gate R3 shows CD41 labelled platelets, Gate R1 shows red blood cells, Gate R4 shows leukocytes and Gate R2 shows background levels. **(a)** Whole Blood. **(b)** PRP spin 1, gate R2 shows high background likely to be from red blood cell lysis. **(c)** PRP spin 2. **(d)** Pelleted platelets.

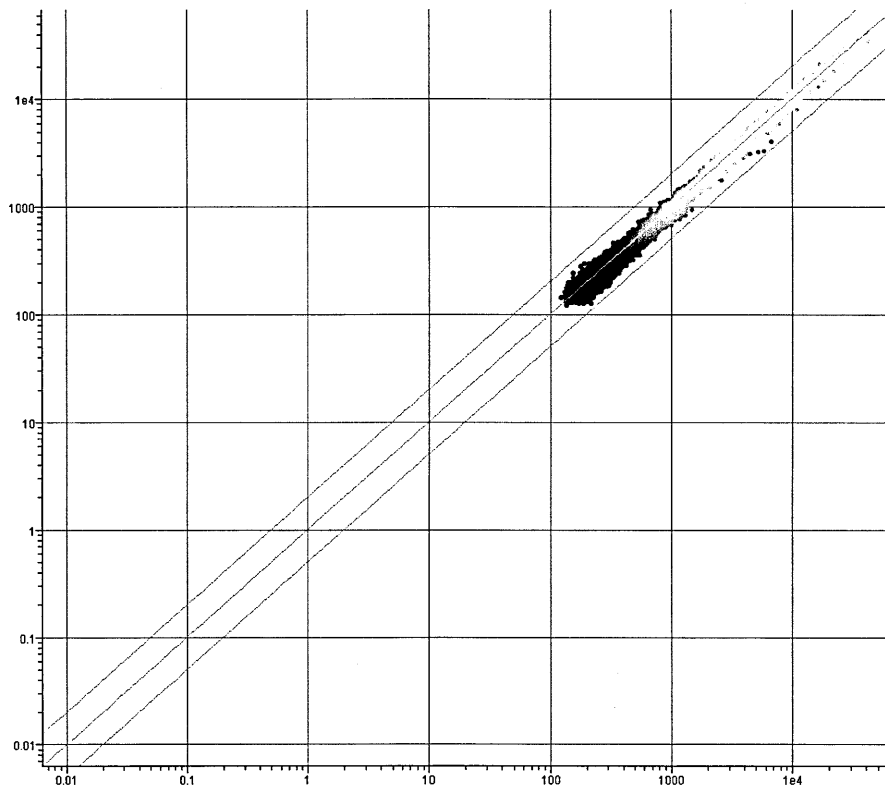
### 5.3.2 cDNA microarray analysis of platelet samples

Total RNA was extracted from the purified platelets of 2 ET patients and one normal control. The purity of the platelet samples to be analysed and the amount of information that can be extracted from this type of experiment was first investigated in a self vs. self microarray experiment using the normal control platelets. This is particularly important given the small amount of RNA and therefore transcripts present in platelets.

### 5.3.2.1 Self v self Platelet cDNA microarray analysis

A self vs self microarray experiment uses the same sample and labels it once with Cy5 and once with Cy3. The two labelled samples are then hybridized to the same microarray slide and analysed as for a patient vs normal control experiment. Normal control platelets isolated from 55ml of peripheral blood were used for this analysis. As shown in Fig 5.3 it can be seen that expression of all genes in the normal control is within the 1:1 ratio. This indicates that there is no differential gene expression between the two labelled samples from the normal control. Markers for platelets and other cell types are present on the array and it is therefore possible to gain some insight into the levels of contaminating cells.  $\beta 2$  integrin, a leukocyte marker, is spotted on the array three times. One spot has a signal intensity value of 300, consistent with background levels, however the remaining two spots show signal levels of 1000. Signal intensity levels of Glycophorin A, a red blood cell marker spotted twice on the array, are around 200-300, again consistent with background fluorescence levels.

The gene with highest expression level in the normal control platelet sample was *MASPI* (mannan-binding lectin serine protease 1), which is an activating component of Ra-reactive factor (RARF). RARF is a complement-dependent bactericidal factor. There are no previous reports of this gene being expressed in human platelets. The highest expressing genes in normal platelets (signal intensity over 25,000) are shown in Table 5.2 This list includes some genes which are also expressed in neutrophil populations, such as *WNT2B* and *INSR*, and also *FCN1* which is expressed in leukocytes. Also included are several ribosomal proteins: *L37*, *S24* and *L10*



**Figure 5.3 Self v Self Platelet Microarray.** Scatterplot of self v self normal control platelet data. Each spot is the expression ratio of a single gene. The green lines represent expression ratios in the normal range. The middle green line shows the value where points would lie if both samples have identical expression ratios.

Platelet specific genes are represented on the array and expression of these is high, although they are not among the highest expressing genes. Platelet factor 4 (*PF4*) intensity 15,570, glycoprotein Iba (*GP1BA*) intensity 2000. To identify the total number of genes expressed in the sample any genes with a signal intensity of less than 500, which is consistent with background signal levels, were excluded. This leaves 5,271 genes present on the array that are expressed in platelets. The total number of cDNAs spotted on the array is 9,932, indicating that 53% of total transcripts on the array are expressed in platelets.

**Table 5.2 Genes with highest expression levels in normal platelets**

<b>Gene Symbol</b>	<b>Full name</b>	<b>function</b>
<b><i>MYBPC3</i></b>	myosin binding protein C	cardiac muscle contraction, protein binding
<b><i>TROAP</i></b>	trophinin associated protein (tastin)	cell adhesion, centrosomes, cytoskeleton
<b><i>SFTPD</i></b>	surfactant protein D	Cell proliferation
<b><i>TRIM27</i></b>	tripartite motif-containing 27	Cell proliferation
<b><i>WNT2B</i></b>	wingless-type MMTV integration site family, member 2B	Cell proliferation
<b><i>MASPI</i></b>	mannan-binding lectin serine peptidase 1 (C4/C2 activating component of Ra-reactive factor)	Complement Activation
<b><i>PTPRO</i></b>	protein tyrosine phosphatase, receptor type, O	Integral membrane protein
<b><i>MYL12B</i></b>	myosin, light chain 12B, regulatory	Motor activity
<b><i>PPBP</i></b>	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7	Platelet derived growth factor, chemokine, immune response, cell division
<b><i>INSR</i></b>	insulin receptor	Regulatory
<b><i>TRAF4</i></b>	tumour necrosis factor receptor-associated factor 4	signal transduction
<b><i>FCN1</i></b>	ficolin (collagen/fibrinogen domain containing)	signal transduction
<b><i>RPL10</i></b>	Human ribosomal protein L10	Translational elongation
<b><i>RPL37</i></b>	ribosomal protein L37	Translational elongation
<b><i>RPS24</i></b>	ribosomal protein S24	Translational elongation

### ***5.3.2.2 cDNA microarray analysis of platelets from patients with ET***

Platelet RNA samples from two ET patients (M1372 and M1377) were used for cDNA microarray analysis and compared against the normal platelet sample used above. Given the large volumes of normal peripheral blood that would be required to generate sufficient RNA to create a normal platelet pool it was not possible to analyse the patient samples against pooled normal control platelets.

When comparing the two patient platelet samples against the normal platelet sample it was possible to identify genes that were differentially expressed in the patients. Excluding any genes specifically expressed in males and females, or duplicated genes, 35 genes were identified as down-regulated by greater than 0.35 fold in both patients. The analysis also identified 37 genes that were up-regulated by greater than 2 fold in both patients. The most up-regulated genes include Ras suppressor protein 1 (*RSUI*), the general transcription factor *GTF2A2*, the anti-apoptotic *DAD-1* (defender against cell death 1), pro-apoptotic *DAP* (Death-associated protein), the non-protein coding gene *DLEU1* (deleted in lymphocytic leukaemia 1), *CCPG1* (cell cycle progression 1), which is involved in cell cycle regulation and *EIF1B* (eukaryotic translation initiation factor 1B) which regulates translation. The most down-regulated genes include *NELL2* (NEL-like 2) which is expressed in haematopoietic cells, *ZNF266* (zinc finger protein 266) which is involved in erythroid and megakaryocyte differentiation, and several ribosomal proteins: S7, L30, L28, P2, L6, L34 and L37.

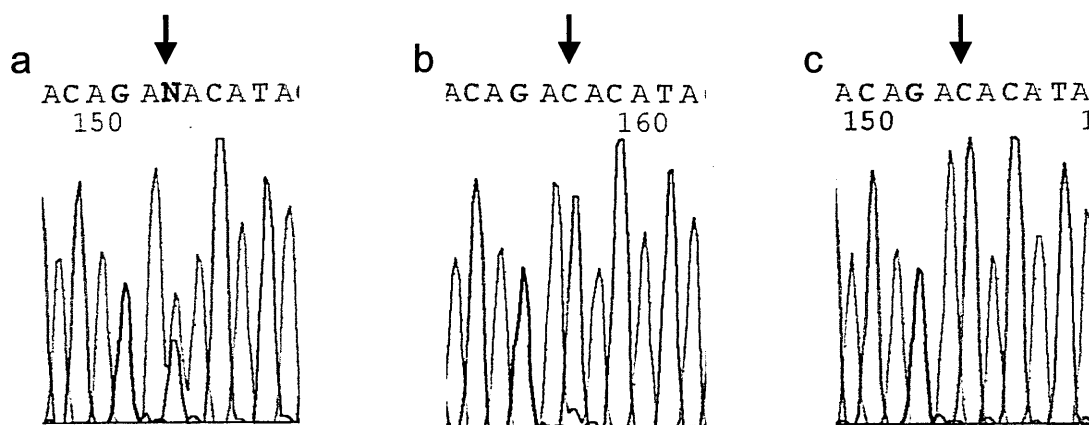
### ***5.3.3 Mutation analysis of JAK2 in neutrophils of ET and PRV patients***

The *JAK2* V617F mutation was studied in all patients detailed in Table 5.1. Exon 14 of *JAK2* was PCR amplified from neutrophil genomic DNA. PCR products were then subjected to cycle sequencing using the same primer set. Differences between the normal *JAK2* sequence and sequences obtained from the samples studied were identified using pairwise analysis of sequences using Sequence Analysis software (Informagen).

Cycle sequencing of genomic DNA confirmed the presence of a G to T point mutation in exon 14 of *JAK2* at nucleotide 1849 in all patients. All patients were heterozygous



for the mutation and it can be seen in fig 5.4 that there appeared to be varying degrees of heterozygosity between the patients, ranging from approximately 62% mutated cells in case M1415 to less than 10% in case M1360. This was calculated from the relative peak heights and can be seen in Fig 5.4. Sequence analysis revealed no further nucleotide changes in these patients.



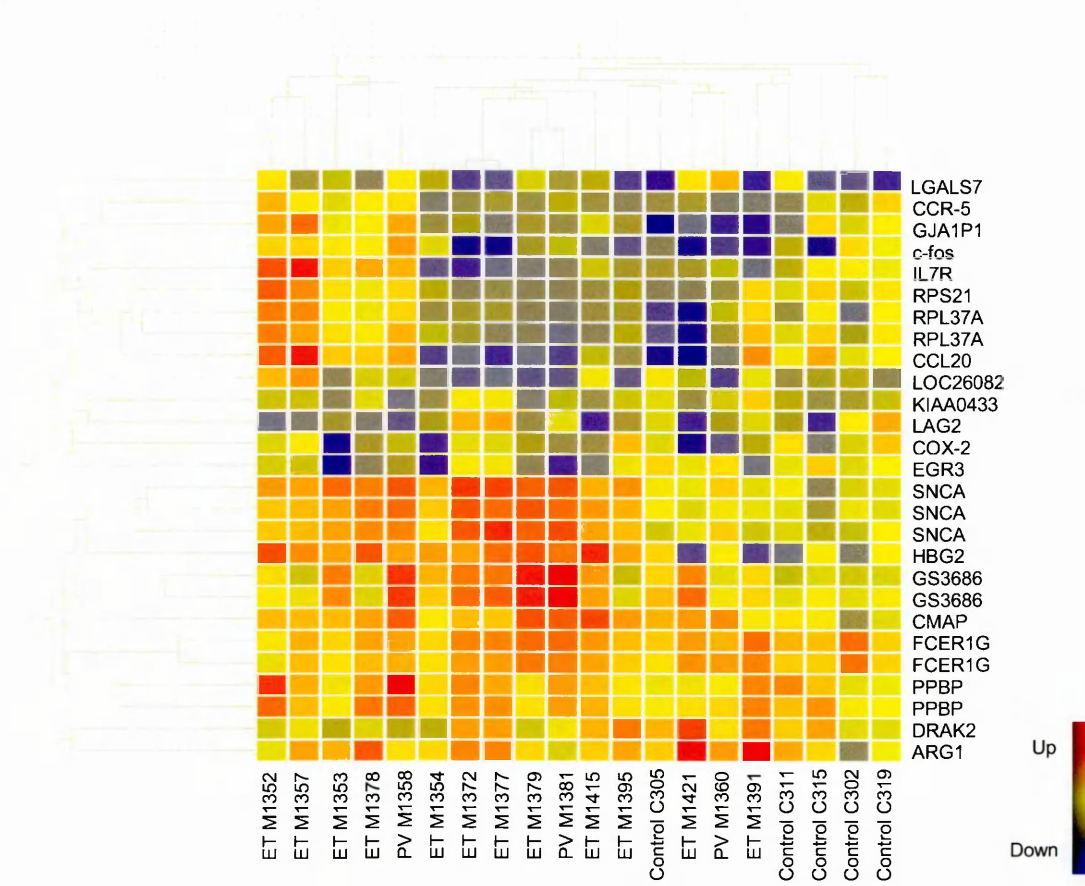
**Figure 5.4 *JAK2* V617F mutation analysis.** Sequencing electropherograms from *JAK2* exon 12 sequencing. The reverse sequencing read is shown and the position of the G to T mutation is indicated with an arrow. (a) Patient M1415 showing approximately 62% mutated cells. (b) Patient M1360 showing less than 10% mutated cells. (c) Normal control neutrophils showing only the wild type sequence.

#### 5.3.4 *cDNA Microarray analysis of neutrophils*

##### 5.3.4.1 *Gene expression analysis of ET and PV*

Neutrophils from *JAK2* V617F positive ET and PV patients were compared with a pool of normal control neutrophils by cDNA microarray. Five individual normal control neutrophil samples were also compared against the normal neutrophil pool. Analysis of these samples using the genespring software identified 8 genes that were up-regulated by greater than 1.6 fold in 5 of 12 ET patients and 13 genes that were down-regulated

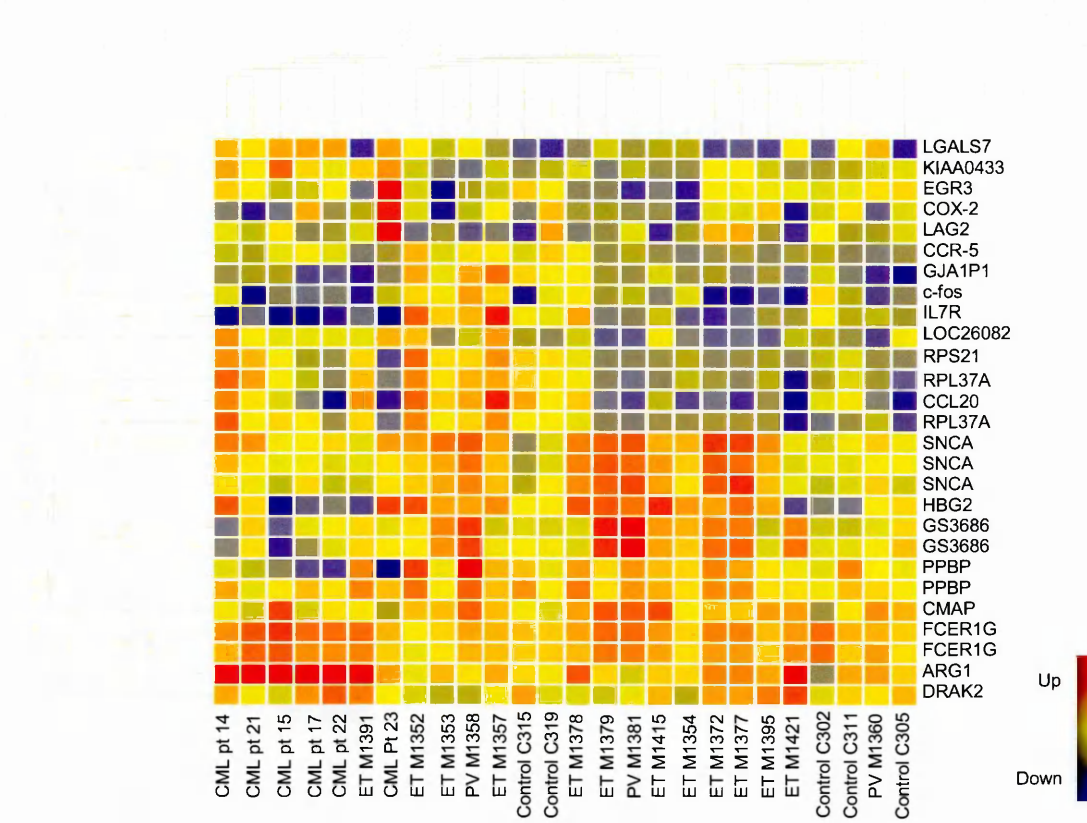
by at least 0.6 in 5 of 12 patients. Using this gene set for hierarchical clustering analysis it can be seen in Fig 5.5 that the normal controls cluster in two separate branches and that one PV and three ET samples also cluster in these branches. The remaining patient samples cluster separately and there appears to be no separation between ET and PV. This indicates that the gene expression profile of neutrophils from ET patients showed a low level of heterogeneity as compared to normal controls. However, it was possible to identify some differentially expressed genes between the ET patient and normal control samples. The most up-regulated genes in ET include *SNCA* (Synuclein, alpha) and *FCER1G* (Fc fragment of IgE). The most down-regulated genes include *EGR3* (Early growth response 3) and *CCR-5* (chemokine receptor 5).



**Figure 5.5 Hierarchical clustering of 21 genes differentially expressed in neutrophils of ET patients.** Each column represents a single patient or control sample on the microarray and each row represents a separate gene. Samples are numbered as in Table 5.1 and C3- samples are normal controls.

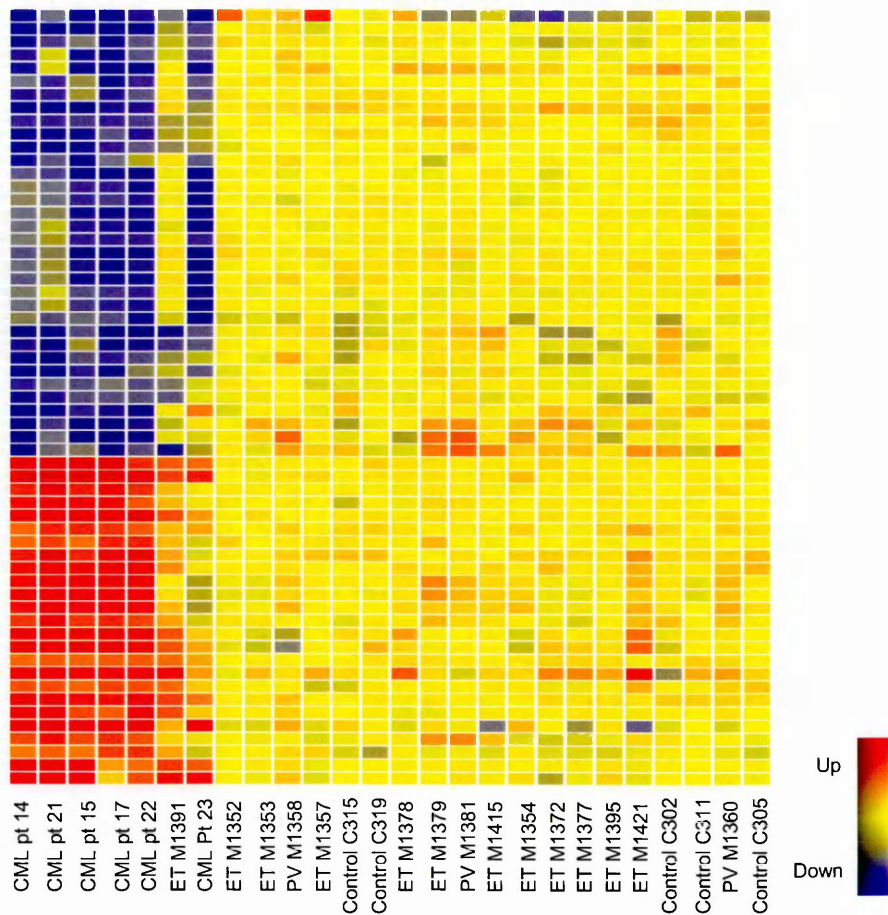
5.3.4.2 Gene expression analysis of ET, PV and CML

Neutrophils were collected from 6 CML CP patients (14, 15, 17, 21, 22 and 23 in Table 3.1) and RNA was extracted for cDNA microarray analysis. When CML, ET, PV and normal controls are analysed with the set of genes identified as differentially expressed in ET the ET, PV and control groups do not separate into distinct branches (Fig 5.6). In contrast the CML patients cluster in a single branch with one patient with ET: M1391. Interestingly, this patient was later to progress to AML.



**Figure 5.6 Hierarchical clustering of 21 genes differentially expressed in neutrophils of ET and CML patients.** Each column represents a single patient or control sample on the microarray and each row represents a separate gene. Samples are numbered as in Table 5.1 and C-3 samples are normal controls. Samples from CML patients are numbered pt 14 – pt 23.

Analysis with the CML patient group identified a set of 59 genes that were either up-regulated by greater than 2 fold in 5 of 6 patients or down regulated by less than 0.5 fold in 5 of 6 cases. Genes up-regulated in CML included eosinophil granule major basic protein (*MBP*), Neutrophil gelatinase-associated lipocalin precursor (*NGAL*), helix-loop-helix protein (*Id-2*) and myeloid-related sequence (*DEFA1*). Down regulated genes in CML included IL-7, hsp70, E-type ATPase (*HB6*) and secreted and transmembrane 1 (*SECTM1*). Using this gene set for hierarchical clustering the CML patients again cluster together as a distinct group (Fig 5.7). With the exception of ET patient M1391, the ET, PV and normal control samples are branched separately from the CML patients. This patient also clustered with the CML patients when the ET gene set was used for analysis.

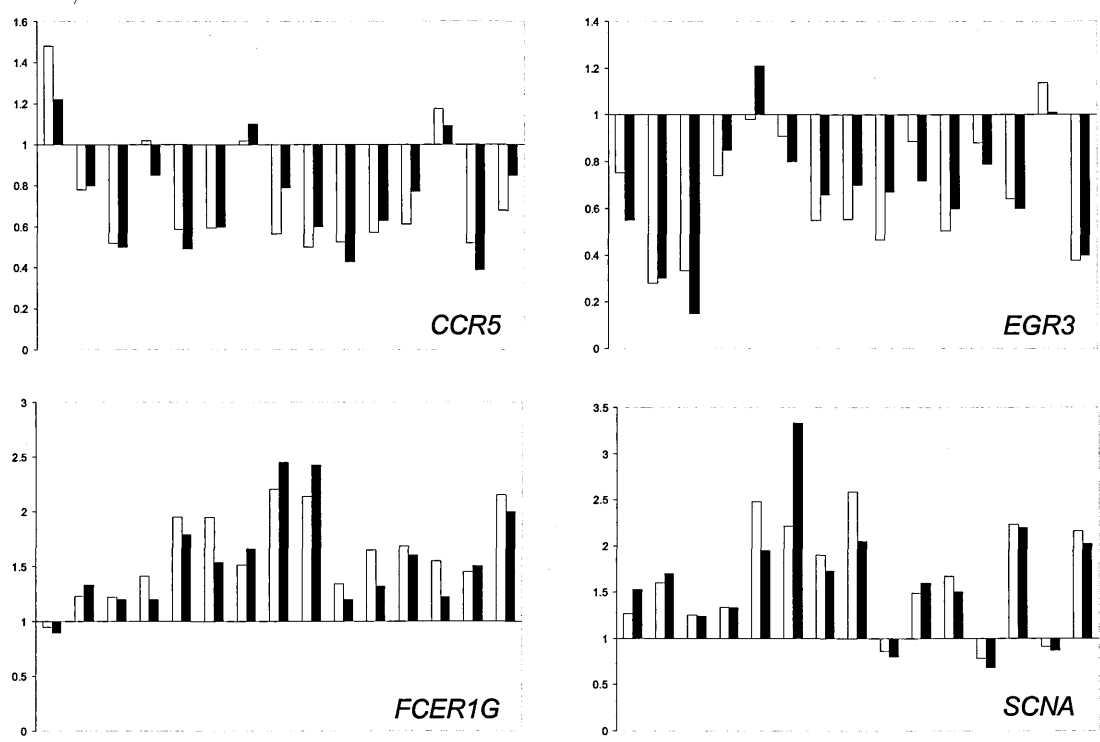


**Figure 5.7 Hierarchical clustering of 59 genes differentially expressed in neutrophils of CML patients.** Each column represents a single patient or control sample on the microarray and each row represents a separate gene. Samples are numbered as in Table 5.1 and C-3 samples are normal controls. Samples from CML patients are numbered pt 14 – pt 23.

### 5.3.5 Confirmation of Gene expression data by Real Time PCR

The expression levels of four differentially expressed genes were measured using real-time PCR to validate the results obtained using the microarray platform. Four genes that were differentially expressed the neutrophils of patients with ET (*SNCA*, *EGR3*, *CCR5*, *FCER1G*) were used for confirmation and the expression data of each gene was

normalized to the endogenous reference  $\beta 2M$ . Expression levels obtained by real time PCR were compared with the microarray data and as can be seen in Fig 5.8 the results correlate well. This confirms that the genes identified as deregulated by the microarray platform have been identified correctly. Statistical analysis performed on results obtained with real-time quantitative PCR in the group of ET patients and controls showed that expression levels of *EGR3* and *FCER1G* were significantly different between patients and normal controls ( $P=.01$  for both genes). However, the changes in gene expression for *SNCA* and *CCR5* were not statistically significant between the patient and control groups.



**Figure 5.8 Confirmation of Gene Expression Data by Real Time PCR.** Gene expression ratios from the microarray data are shown as white bars and ratios from real time PCR are shown as black bars. The data shown is from the patient neutrophil samples detailed in Table 5.1.

## 5.4 Discussion

A pure population of platelets has been isolated from the peripheral blood of patients with ET and from normal controls. Analysis by flow cytometry indicates that contaminating levels of other blood cell types is low. When the platelet samples are analysed by cDNA microarray it was noted that the expression of Glycophorin A was extremely low, indicating that there is minimal red cell contamination in the samples. In addition to this it is worth noting that less than 1% of circulating red blood cells contain residual RNA so this is unlikely to affect the results in the present study. However, levels of two of the three spots of  $\beta 2$  integrin, a leukocyte marker are above background levels. It is possible that some contaminating leukocytes may be present in the sample. However it has been shown that activated platelets also express  $\beta 2$  integrin and resting platelets express it at low levels (Philippeaux *et al.* 1996). It may therefore be the case that the expression of  $\beta 2$  integrin detected by microarray is from the platelets and not contaminating leukocytes. This is in agreement with the flow cytometry data. Therefore, it may be concluded that a pure population of platelets has been isolated from the samples included in this study.

Previous studies investigating gene expression in platelet samples estimate that between 13 and 31% of genes are expressed in platelets. These studies show that some of the most abundant transcripts are platelet associated genes such as Platelet factor 4 (*PF4*) and glycoprotein Iba (*GP1BA*). These genes were also expressed at high levels in the current study. The gene with highest expression in the normal platelet sample was *MASPI* (Mannose-binding lectin-associated serine protease), a serine protease that has a role in complement activation (Dobo *et al.* 2009). It has also been identified as being involved in coagulation as it can cleave the thrombin substrates fibrinogen and factor



XIII (Hajela *et al.* 2002). It is thought that expression of *MASPI* plays an important role in the defense against pathogens (Krarup *et al.* 2008). Platelets become stimulated when pathogens are identified and can accumulate at sites of infection so expression of *MASPI* may be important for this function (Yeaman 2010). There were also genes involved in cytoskeletal organisation such as *TROAP* which has been identified in the current study. Cytoskeletal organisation is important for platelet activation and function with reorganisation of the cytoskeleton taking place to allow cell spreading at wound sites (Hartwig *et al.* 1999). Additionally, ribosomal proteins L37, S24 and L10 were highly expressed. This correlates with the fact that platelets can perform protein synthesis and would require an active ribosome to catalyse protein synthesis.

Gene expression profiling of platelet samples from ET patients revealed that compared to a normal control both patient samples had common genes that were differentially expressed. This was the first study to examine ET patient platelet samples by cDNA microarray analysis. Genes with low expression in both patient samples include ribosomal proteins S7, L30, L28, P2, L6, L34 and L37 which are involved in protein production suggesting that in these patients protein production may be reduced. Adding to the sample number would increase the amount of information and allow identification of transcripts that are consistently differentially expressed in platelets of patients with ET. It shows that this type of study would be feasible and that the advent of new platelet specific arrays is likely to reveal insights into the importance of gene expression profiling in platelets. Recently, Gnatenko *et al.* have developed a platelet specific gene chip and have carried out gene expression analysis of ET patients (Gnatenko *et al.* 2010). They reveal a set of 11 transcripts that can identify patients with ET and just 4 of these can predict *JAK2* wild type ET demonstrating the value of platelet RNA profiling.



Presence of the *JAK2* V617F mutation was confirmed by cycle sequencing in all patients included in this study. Gene expression profiling of *JAK2* V617F positive neutrophils in ET and PV reveals that these patients do not have a heterogeneous expression profile when compared to normal control neutrophils. The genes identified as differentially expressed in these patients were seen as deregulated in less than half of the patient samples (5 of 12). This suggests that regardless of the presence of the *JAK2* V617F mutation the neutrophils of these patients exhibit a near normal gene expression profile. Despite this it was possible to identify some differentially expressed genes in the patient and control samples.

Genes with increased expression in the neutrophils of ET patients included *SCNA* (Synuclein Alpha). This gene is associated with Parkinson disease and dementia and the protein product accumulates in the nervous systems of patients with the disease (Goedert 2001). Expression of the gene has been detected in platelets, lymphocytes and erythrocytes suggesting some as yet unknown function in the haematopoietic system (Nakai *et al.* 2007). Over expression of *SCNA* in cell lines can result in increased proliferation (Lee *et al.* 2003). Increased expression of this gene in ET may contribute to an increase in proliferation of neutrophils.

*FCER1G* (Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide) expression was increased in the neutrophils of ET patients in the current study. *FCER1G* is an FC receptor chain that, in cooperation with other protein kinases, mediates intracellular signaling (Nambiar *et al.* 2003). It is also involved in allergic reaction (Pawankar 2001). Increased expression of this gene has previously been reported in patients with PV (Pellagatti *et al.* 2003).

Expression of *EGR3* was significantly decreased in the neutrophils of *JAK2* mutated ET patients. *EGR3* (Early Growth Response 3) is a transcription factor that increases transcription of the Fas ligand (Mittelstadt and Ashwell 1998). If expression of *EGR3* is decreased this may then result in decreased expression of the Fas ligand (Yasunaga *et al.* 2004). When Fas ligand expression is reduced in inflammatory neutrophils the viability of these neutrophils is increased (Jonsson *et al.* 2005). It could therefore be suggested that a decrease in *EGR3* expression in neutrophils of patients with ET could act through a decrease in Fas ligand expression to increase neutrophil viability in these patients.

CC Chemokine Receptor 5 (*CCR5*) expression was reduced in ET patients in the current study. *CCR5* is a G protein coupled receptor that is involved in inflammation and autoimmunity (Ariel *et al.* 2006). Interestingly, *CCR5* is an upstream activator of *JAK2* (Wong *et al.* 2001; Mueller and Strange 2004). Given that the *JAK2* V617F mutation results in a constitutively active *JAK2* kinase a decrease in *CCR5* expression in ET patients with the mutation is unlikely to have an effect on activation of *JAK2*.

A study by Puigdecanet *et al.* used whole genome oligonucleotide microarrays to analyse gene expression profiles of neutrophils of 21 *JAK2* mutated and 19 *JAK2* wild type ET patients (Puigdecanet *et al.* 2008). They demonstrated that expression of genes involved in the immune response was increased in the neutrophils of all ET patients. These include genes involved in neutrophil chemotaxis such as *PPBP*, which was also increased in the ET neutrophils analysed in the current study. *CCL20* expression was also increased in the study by Puigdecanet but was decreased in patients in the current study. This may be due to platform variation and also to the increased sample size used by Puigdecanet. They identified genes that were differentially expressed between

patients with and without the *JAK2* V617F mutation but noted that expression of these genes were not significantly different to normal controls. They identified the genes *FOSB* and *CISH* as predictors of *JAK2* mutation status in neutrophils. *CISH* expression is decreased in *JAK2* wild type patients and *FOSB* is increased in these patients. Expression levels of *FOSB* in the patients in the current study were within the range observed for the normal controls. *CISH* is not present on the microarrays used so it is not possible to comment on expression of this gene.

A further study investigating gene expression in the CD34<sup>+</sup> cells of ET patients showed that the gene expression profile of these cells could not distinguish between patients with mutated or wild type *JAK2* (Catani *et al.* 2009). It was also demonstrated that gene expression was not significantly altered between patients and normal controls. These results suggest that secondary molecular aberrations could be responsible for the pathogenesis of ET.

Gene expression profiling of CML neutrophils revealed that these cells were clearly highly heterogeneous when compared to normal and *JAK2* V617F positive samples. Although the *JAK2* mutation is detected in the neutrophils of the patients included in this study it does not appear that the resulting activated kinase produces a deregulated gene expression profile similar to that which is observed in CML. This provides further evidence that the *JAK2* V617F neutrophils of ET and PV patients are more similar to normal neutrophils despite the presence of the mutation. The patient M1391 that clustered with the CML patients clearly showed a distinct gene expression profile from the other ET and PV samples. This patient was to progress from ET to AML suggesting that the gene expression in this patient was becoming more like an acute leukaemia. The difference seen in the expression profile of this patient suggests that a secondary

genetic change may have occurred to result in this deregulated expression pattern and to cause the progression of the disease. Further cytogenetic profiling or SNP array analysis of this sample could identify subsequent changes which may have led to disease progression. In fact, it has been shown that expression of *JAK2* V617F can result in an increase in DNA damage and genetic instability and it is believed that this results in disease progression (Plo *et al.* 2008). Gene expression profiling of neutrophils could prove be a useful tool to predict disease transformation in patients with ET, however a larger sample size would reveal the true power of this prediction.

The data presented here demonstrate the feasibility of platelet gene expression profiling and suggests that platelet profiling could reveal some novel insights into the pathophysiology of ET. Gene expression profiling of neutrophils of ET patients shows that gene expression changes are observed between patients and normal controls but are uncommon despite the presence of the *JAK2* V617F mutation. Gene expression profiling of neutrophils of ET patients may be able to predict transformation to AML but a larger sample size is needed to confirm this result.

***Chapter 6***

***Gene Expression Profiling of  
Telomerase Related Genes in  
Myelodysplastic Syndromes***

## **6.1 Introduction**

### **6.1.1 Pathogenesis of MDS**

The Myelodysplastic Syndromes (MDS) are a heterogeneous group of haematopoietic malignancies characterised by ineffective haematopoiesis and dysplastic bone marrow (Nimer 2008). There is dysplasia in the bone marrow of at least one of the myeloid, erythroid or megakaryocyte lineage with peripheral blood cytopaenia (Scott and Deeg 2010). MDS is one of the most common cancers in patients over 70 years of age and onset before age 50 is rare (Rollison *et al.* 2008). In early MDS dysplastic clones will proliferate but this is mediated by an increase in apoptosis resulting in hypercellular bone marrow with peripheral blood cytopaenia (Nimer 2008; Davids and Steensma 2010). In around 30% of patients the disease will progress to Acute Myeloid Leukaemia (Mufti *et al.* 2008). Disease progression is thought to be mediated by several different pathways. The anti-apoptotic protein BCL-2 is thought to play a role in promoting transformation by preventing apoptosis of the dysplastic clone (Parker *et al.* 2000). Gene expression profiling shows that in advanced stages of disease patients have deregulated expression of genes involved in the DNA damage response and cell cycle checkpoints which may confer a growth advantage to these cells (Pellagatti *et al.* 2010). MDS can occur as a secondary complication after radiotherapy or chemotherapy and a high percentage of these patients have deletion of the long arm of chromosomes 5 or 7 (Le Beau *et al.* 1986). This therapy related MDS, or tMDS has poor prognosis and five year survival has been reported at less than 10% (Smith *et al.* 2003).

The MDS patients in the current study have been classified according to the FAB (French American British) classification (Bennett *et al.* 1982). According to this

classification refractory anaemia (RA) and refractory anaemia with ringed sideroblasts (RARS) are associated with low risk disease and both have less than 5% blasts in the bone marrow with RARS exhibiting  $\geq 15\%$  ring sideroblasts. Refractory anaemia with excess blasts (RAEB) is associated with progression to AML. The patients with RAEB were divided into two groups: RAEB-1 with less than 10% blasts in the bone marrow and RAEB-2 with 10 to 19% blasts in the bone marrow. Prognosis of MDS is evaluated using the International Prognostic Scoring System (IPSS). Using this scoring system risk is calculated based on blast percentage, number of cytopaenias and bone marrow cytogenetics (Greenberg *et al.* 1997). Table 6.1 shows how the IPSS score is calculated.

**International Prognostic Scoring System in MDS**

Prognostic Variable	Score Value				
	0	0.5	1.0	1.5	2.0
BM Blast %	<5	5-10	-	11-20	21-30
Karyotype	Good	Intermediate	Poor		
Cytopaenias	0/1	2/3			

**Table 6.1 IPSS in MDS.** Good karyotype is normal, -Y, del(5q) and del(20q). Poor karyotype is complex with  $\geq 3$  abnormalities. Intermediate karyotype is other abnormalities. Low risk is a score of 0, Intermediate-1 0.5-1.0, Intermediate-2 1.5-2.0 and High risk is  $\geq 2.5$ . (Greenberg *et al.* 1997)

All MDS patients require supportive care in the form of transfusion of red blood cells and platelets (Greenberg 2010). The thalidomide analogue lenalidomide has been used to successfully treat low risk MDS patients with del(5q) and it has been reported that up to 67% of patients achieve transfusion independence and 73% achieve cytogenetic response (List *et al.* 2006). In low risk patients without del(5q) only 26% of patients were transfusion independent after treatment (List *et al.* 2006). However, the drug has

not yet been approved for use in Europe due to concerns over increased risk of progression to AML (Garcia-Manero and Fenaux 2011). For patients with high risk MDS the only curative treatment is by haematopoietic stem cell transplant and up to 29% of these patients will have 5 year disease free survival. This increases to 41% in patients with RA/RARS (Chang *et al.* 2007).

Between 40-50% of patients with RAEB will transform to AML and only 5-15% of RA/RARS patients (Greenberg *et al.* 2002). Gene expression profiling has shown that approximately 23% of MDS patients exhibit an AML-like gene expression profile (Mills *et al.* 2009). This profile carries a high risk of leukaemic transformation to AML. The most frequent genetic abnormality in de novo AML is mutation of exon 12 of Nucleophosmin (*NPM1*) (Falini *et al.* 2005). *NPM1* is a ubiquitously expressed shuttling protein with nucleolar localisation that acts as a molecular chaperone (Borer *et al.* 1989; Okuwaki *et al.* 2001). The mutations of *NPM1* characterised by Falini *et al.* are in exon 12 of *NPM1* the most frequent of which is a duplication of a TCTG tetranucleotide that results in a frame shift that replaces the last 7 amino acids of the C-terminus of the protein. The other mutations detected by Falini *et al.* are insertions or multiple base substitutions and result in the same mutant C-terminal sequence. The mutations are most commonly found in normal karyotype AML with up to 60% of these patients having mutation of *NPM1* (Falini *et al.* 2005). Patients with mutation have higher rates of complete remission and survival compared to those without the mutation (Schnittger *et al.* 2005). These mutations cause *NPM1* to localise to the cytoplasm rather than the nucleus. The mutation is thought to contribute to leukaemogenesis by increasing protein levels of c-MYC and stabilising the protein (Bonetti *et al.* 2008). The mutant also regulates the ARF-p53 tumour-suppressor pathway and can prevent initiation of p53 (Colombo *et al.* 2006). Additionally, recent findings have shown that



NPM1 can bind DNA that forms G-quadruplexes (Federici *et al.* 2010). G-quadruplexes are a four stranded helical structure formed from G rich DNA sequences (Phan 2010). It has been suggested that NPM1 may be involved in transcriptional regulation of genes that have these G-quadruplex sequences at their promoters (Federici *et al.* 2010). Genes with promoters that can form these structures include the *hTERT* activator *c-MYC* (Siddiqui-Jain *et al.* 2002). G-quadruplexes can also be found at the single strand overhang of telomeres and may play a role in preventing telomerase from elongating the telomeres (Maiti 2010). The study by Federici *et al.* (Federici *et al.* 2010) reports that the *NPM1* mutations reported in AML patients disrupt the C terminal G-quadruplex binding domain of the protein. This implies that any binding activity of NPM1 would be reduced by the mutation and authors suggest this may drive disease progression by promoting gene deregulation. Given the high percentage of MDS patients that transform to AML it will be of interest to investigate the role of *NPM1* mutations in disease progression of patients with MDS.

### ***6.1.2 Karyotypic abnormalities in MDS***

In primary MDS karyotypic abnormalities are identified in 45% of patients and occur most frequently in patients with RAEB (Pozdnyakova *et al.* 2008). Translocations are rare in MDS and any karyotype abnormalities typically consist of partial or complete deletion or addition of chromosome regions (Fenaux 2001). The most commonly reported cytogenetic abnormalities in MDS are del(5q), del(7q), trisomy 8, del(20q) and -Y (Haase *et al.* 2007). A more complex karyotype leads to a worse prognosis for the patient. For example, patients with normal karyotype have a median survival of 53 months, with 1 or 2 abnormalities survival is 35-38 months but with 3 abnormalities this

decreases to 17 months and with 4 to 6 abnormalities median survival is only 9 months (Haase *et al.* 2007).

Patients with 5q- syndrome have del(5q) as the sole karyotypic abnormality. These patients have distinct clinical characteristics including macrocytic anaemia, thrombocytosis, dyserythropoiesis and megakaryocytes with nonlobulated nuclei (Sokal *et al.* 1975; Boulton *et al.* 2010). The disease is slow to develop, is associated with good prognosis, indolent disease phenotype and patients respond well to treatment with lenalidomide (Giagounidis *et al.* 2004; Mohamedali and Mufti 2009). In a recent study of 541 patients with a deletion of 5q Mallo *et al.* demonstrated that there is no significant difference in overall survival between those with del(5q) as the sole abnormality and those with del(5q) and one additional abnormality (Mallo *et al.* 2011). However, they did demonstrate that one additional abnormality gives a higher risk for progression to AML and that with two or more additional abnormalities both overall survival and risk of AML transformation are significantly increased.

Approximately 5% of patients who develop de novo MDS have del(7q) as the sole karyotypic abnormality. This is associated with poor prognosis (Olney and Le Beau 2001). Patients with del(7q) tend to have better survival than patients with monosomy 7 but both abnormalities are still considered to confer poor prognosis (Haase *et al.* 2007). Trisomy 8 in MDS is seen in 8% of patients and is associated with intermediate risk disease (Haase *et al.* 2007). The del(20q) abnormality is observed in approximately 5% of MDS cases and when present as the sole abnormality is associated with low risk disease, low risk of progression to AML and longer than average survival (Wattel *et al.* 1993). However, when del(20q) is part of a complex karyotype patients have decreased survival (Olney and Le Beau 2001).

Y chromosome loss is considered a normal age related abnormality but in cases where every cell is identified as having loss of the Y chromosome there is a significant increase in MDS/AML (Wong *et al.* 2008). However, it is thought to be unlikely that Y chromosome loss is causing MDS/AML but instead the MDS/AML occurs after a mutation in a -Y cell that will proliferate to such an extent that any cells with the Y chromosome will be eliminated (Wong *et al.* 2008).

Recently gene expression profiling has been used to show that certain karyotypic abnormalities in MDS have been associated with changes in gene expression of distinct pathways (Pellagatti *et al.* 2010). Patients with trisomy 8 exhibit deregulation of pathways of the immune response, patients with del(7q) deregulate pathways involved in cell survival, and patients with del(5q) show deregulation of integrin signalling and cell cycle regulation pathways.

### **6.1.3 Genetic Abnormalities in MDS**

There are several genetic abnormalities that are common amongst MDS patients. Some of the most frequent point mutations observed in MDS are in the Runt-related transcription factor 1 (*RUNX1*) gene. Mutations in this gene are found in up to 20% of patients and are more common in those patients with tMDS (Harada and Harada 2009). Patients with the mutation have a significantly worse prognosis than those without mutation (Harada *et al.* 2004). It is thought that mutation reduces *RUNX1* activity and initiates MDS by inhibiting differentiation of haematopoietic stem cells (Harada and Harada 2011).

Mutations of the p53 tumour suppressor are present in over 20% of high risk MDS patients and are more common in advanced disease (Sugimoto *et al.* 1993; Adamson *et*

*al.* 1995; Davids and Steensma 2010). In low and intermediate-1 risk patients with del(5q) the mutation has been detected in 18% of patients and is associated with progression to AML (Jadersten *et al.* 2011). The mutation is common in patients with tMDS and mutation is strongly associated with complex karyotype, deletion of 5q and poor prognosis (Christiansen *et al.* 2001).

Other common abnormalities in MDS include mutation of the ten-eleven translocation 2 (*TET2*) gene, the *RAS* oncogene and the chromatin binding activator of the retinoic acid receptor *ASXL1* (Neubauer *et al.* 1994; Padua *et al.* 1998; Kosmider *et al.* 2009; Thol *et al.* 2011). The number of molecular and cytogenetic abnormalities that have been described in MDS patients indicates the complexity of the disease and further investigation of these aspects is warranted to increase understanding of disease evolution and prognosis.

#### **6.1.4 Telomeres and Telomerase in MDS**

Telomere length in MDS is heterogeneous in bone marrow samples but shortened telomeres have been associated with poor prognosis, leukaemic transformation and higher risk disease (Ohyashiki *et al.* 1994; Boultonwood *et al.* 1997; Ohyashiki *et al.* 1999; Sieglöva *et al.* 2004). In CD34<sup>+</sup> cells telomere length is decreased in patients when compared to normal controls particularly in cases with unfavourable IPSS scores (Engelhardt *et al.* 2000; Rigolin *et al.* 2004). Shortened telomeres are often linked to complex karyotypic abnormalities suggesting that reduced telomere length leads to genomic instability and poor prognosis (Ohyashiki *et al.* 1994; Boultonwood *et al.* 1997; Ohyashiki *et al.* 2001). Recently Lange *et al.* have shown that it is possible to measure individual telomere lengths in MDS (Lange *et al.* 2010). They used a novel technique

known as Telomere/Centromere-Fluorescence In Situ Hybridisation (T/C-FISH). This involves a FISH probe against telomere repeats used in conjunction with fluorescence R-banding to measure the telomere length of each individual chromosome. This identified certain subtypes of MDS that have stabilized or even increased telomere length. Patients with monosomy 7 showed significantly longer telomeres than normal karyotype patients and this was thought to be directly caused by telomerase activation in these patients. In a subsequent study this group also demonstrated that patients with a del(5q) being treated with lenalidomide who underwent disease transformation had significantly shorter telomeres than those who achieved cytogenetic response (Gohring *et al.* 2011). In patients who responded to treatment telomere length was not significantly different from controls and this again suggests that shortened telomeres can predict leukaemic transformation.

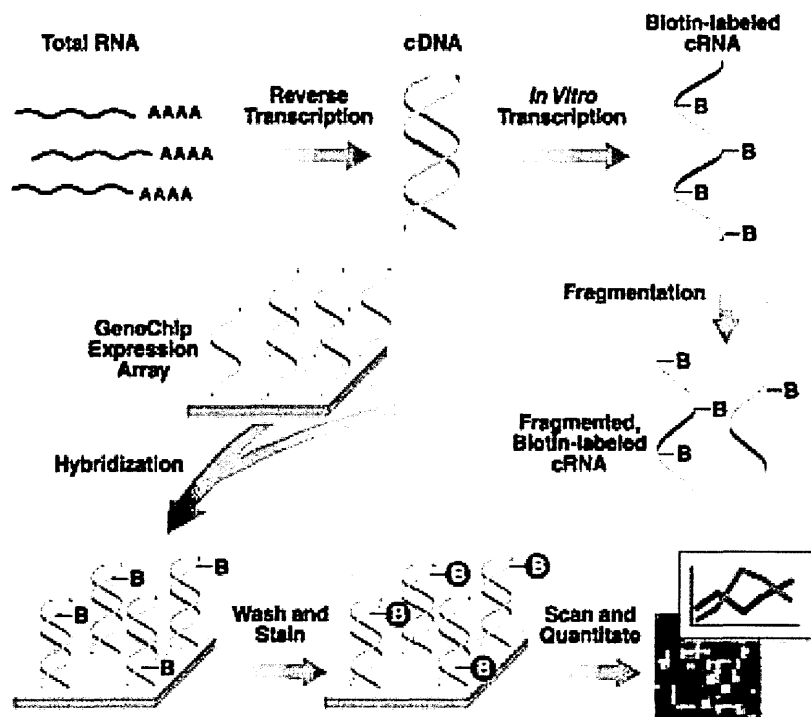
Accelerated telomere shortening can be predictive of tMDS. This was suggested by Fern *et al.* who analysed telomere length in patients treated for non haematological malignancies with conventional chemotherapy (Fern *et al.* 2004). They showed accelerated telomere shortening after chemotherapy and a switch to clonal haematopoiesis suggesting a link with tMDS. Autologous haematopoietic stem cell transplantation (aHCT) is used as a treatment for Hodgkin's and non Hodgkin's lymphoma that has the complication of tMDS. Patients who have received aHCT have an initial increase in telomere length after transplant but this is quickly reduced as telomere shortening accelerates in tMDS patients (Bhatia *et al.* 2005; Chakraborty *et al.* 2009). In these patients tMDS can be predicted by increased telomere shortening and it is suggested that this leads to genomic instability that contributes to leukaemic transformation (Chakraborty *et al.* 2009).

Telomerase activity in bone marrow is variable in MDS but it is generally higher in patients than in normal bone marrow (Counter *et al.* 1995; Li *et al.* 2000). In agreement with this telomerase activity has also been reported as increased in CD34<sup>+</sup> cells from MDS patients (Engelhardt *et al.* 2000). A study of telomerase activity in bone marrow mononuclear cells showed that those MDS patients with poor prognosis had high telomerase activity (Fu and Chen 2002). Gurkan *et al.* measured telomerase activity in peripheral blood mononuclear cells of patients with MDS and showed that there was no statistically significant difference in telomerase activity when compared with normal controls (Gurkan *et al.* 2005). However, they did report inferior survival in those patients with higher telomerase activity. More recently expression of *c-MYC*, *hTERT* and telomerase activity has been investigated in the bone marrow of patients with MDS and it has been shown that both *hTERT* and telomerase activity were increased in MDS relative to normal controls but that *c-MYC* was not increased (Briatore *et al.* 2009). Additionally, patients with aggressive disease had significantly higher expression of *hTERT* than those with more favourable disease. This suggests that shortened telomeres and an increase in telomerase activity/*hTERT* expression are associated with disease progression in MDS.

Mutations in the coding regions of both *hTERT* and *hTR* have been identified in cases of familial MDS (Kirwan *et al.* 2009). These mutations can decrease telomerase activity when present and the patients have decreased telomere length. This indicates that telomerase can play a role in the development of MDS and further investigation of the role of telomerase in de novo MDS is warranted.

### 6.1.5 The affymetrix GeneChip platform

The Affymetrix GeneChip platform provides a powerful tool to analyse global gene expression patterns in biological samples. These are synthetic oligonucleotide microarrays. The arrays are constructed on quartz wafers and oligos are constructed on the arrays by photolithography which is the process of light-activated coupling of a nucleotide to a growing chain anchored on the wafer (Dalma-Weiszhausz *et al.* 2006). The expression level of every gene represented on the array is measured by 11 pairs of oligonucleotide probes. The probes are selected from the 3' end of the consensus transcript for each sequence. Each pair of oligos consists of a perfect match (PM) oligo and a mismatch (MM) oligo that differs at nucleotide 13 of 25 and acts as a hybridisation control. The PM oligo directly measures expression of the gene of interest whereas the MM oligo detects any background non-specific hybridisation. The specific intensity of the probe sets are then calculated by subtracting the value of the MM probe from the PM probe. Total RNA is extracted from patient and control samples then amplified using a T7 based linear amplification. Biotin labelled cRNA is produced by in vitro transcription and one sample is hybridised to a single chip. Each chip is washed and stained then scanned to measure fluorescence intensity. An overview of the experimental process is shown in Fig 6.1. The Affymetrix GeneChip Human Genome U133 Plus 2.0 array has been used in the current study and represents over 47,000 transcripts corresponding to 39,000 human genes.



**Figure 6.1 Overview of an Affymetrix Gene Chip experiment.** Image reproduced from the Affymetrix website.

#### 6.1.6 Aims of the study

The aim of this study was to develop a comprehensive list of telomerase specific genes in order to detect genes that are deregulated and may have prognostic value in MDS. This would allow investigation of telomerase specific gene expression profiles in MDS in the hope of identifying specific gene expression profiles for specific patient groups. The *NPM1* gene will also be sequenced in MDS patients to reveal if mutation is present and if it gives any prognostic clues to progression of the disease to AML.



## **6.2 Materials and Methods**

### **6.2.1 Samples**

For gene expression analysis bone marrow samples were obtained from patient and normal control samples and CD34<sup>+</sup> cells were isolated as described in Chapter 2. RNA was extracted using the TRIZOL reagent.

For sequence analysis of *NPM1* peripheral blood was obtained from patient and normal control samples and DNA was extracted using the Qiagen blood maxi kit as described in chapter 2. For sequence analysis of *TRF2* genomic DNA was isolated from CD34<sup>+</sup> cells.

### **6.2.2 Affymetrix Microarray Experiments**

Affymetrix experiments were carried out by Andrea Pellagatti. The Two-Cycle cDNA Synthesis and the Two-Cycle Target Labelling and Control Reagent packages (Affymetrix, Santa Clara, CA) were used to amplify and label 50 ng of total RNA from each sample. This produces biotin labelled cRNA and 20 mg of biotin-labelled cRNA was fragmented. 10 mg of this fragmented cRNA was hybridised to GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix) at 45°C for 16 hr in the Hybridisation Oven 640 (Affymetrix). The arrays were then washed and stained in the Fluidics Station 450 (Affymetrix) and scanned with a GeneChip Scanner 3000 (Affymetrix).

GeneChip Operating Software (GCOS) was used to carry out cell intensity calculation and scaling. Data was analysed using GeneSpring 7.2 software (Agilent Technologies). Signal intensities of all arrays were scaled to a target of 100 and quality control was

carried out using GCOS. Scale factors for all samples were comparable and <3.0. Background levels, percentage of present calls and intensities of eukaryotic hybridisation controls were within the satisfactory range for all samples. The 3’/5’ GAPDH ratio was <3.0 for all samples. Affymetrix CEL files were subjected to Robust Multi-array Average (RMA) analysis (Irizarry *et al.* 2003). To calculate expression ratios for each gene the raw signal of each gene for every MDS patient was divided by the median raw signal of the gene in normal controls. Statistical analysis with GeneSpring was performed on log-transformed data using Welch's approximate t-test or ANOVA and Benjamini-Hochberg multiple testing correction to control the false discovery rate (Benjamini and Hochberg 1995). Hierarchical clustering was calculated using standard correlation.

**6.2.3 Cycle sequencing**

Cycle sequencing was carried out as described in Chapter 2. Primers to amplify *TRF2* were designed by means of the Primer 3 software to amplify the appropriate exons. Primers for *NPM1* were used as described by Falini *et al.* (Falini *et al.* 2005). The primers used for PCR were subsequently used to sequence the resulting PCR product. Table 6.2 shows details of the primer sets used.

**Table 6.2 PCR and Sequencing primers for *TRF2* and *NPM1***

Primer name	Sequence 5’-3’	Annealing Temp °C
<i>TRF2</i> Exon3 Forward	TTGCATGCTCCATTTCATA	62
<i>TRF2</i> Exon3 Reverse	TCAAAGACCCTTGATTTTGG	62
<i>TRF2</i> Exon6 Forward	TTCTTGGTGAAAGAAGCAGAGA	62
<i>TRF2</i> Exon6 Reverse	CAGCGACCACCCTAGTGTTT	62
<i>NPM1</i> Exon12 Forward	TTAACTCTCTGGTGGTAGAATGAA	60
<i>NPM1</i> Exon 12 Reverse	CAAGACTATTTGCCATTCCTAAC	60

## 6.3 Results

### 6.3.1 Identification of a telomerase specific gene list

In order to identify if telomere related genes could be implicated in the pathogenesis of MDS a comprehensive set of genes related to telomerase and telomere structure and function was compiled. This list was generated by performing a thorough search of the current literature and searching the NCBI Gene resource to identify genes with functions related to telomerase or telomeres. This resulted in a list of 82 telomere related genes shown in table 6.3.

**Table 6.3 Telomere related genes used for microarray analysis of MDS patients.**

Gene Name	Function
<i>ACD</i>	TINT1 localised to telomeres via TIN2, functions as a negative regulator of telomerase-mediated telomere elongation
<i>AKT</i>	associates with HSP90, maintains telomerase activity
<i>BIRC5</i>	survivin enhances telomere activity
<i>BLM</i>	recombination mediated telomere lengthening
<i>BRCA1</i>	telomere protection
<i>C15orf20</i>	Pif1 may regulate telomere elongation by decreasing telomerase processivity
<i>CDKN1B</i>	downregulates telomerase activity
<i>CIB1</i>	KIP has a role in telomere length maintenance and regulation
<i>CLK2</i>	hCLK2 has a role in cell cycle progression, apoptosis, and telomere length regulation
<i>c-MYC</i>	increases <i>hTERT</i> transcription
<i>CRM1</i>	enhances nuclear export of TERT
<i>DCLRE1B</i>	protection of telomeres
<i>DKC1</i>	telomerase subunit
<i>EGFR</i>	Antisense epidermal growth factor receptor RNA transfection in human glioblastoma cells down-regulates telomerase activity and telomere length
<i>ERCC1</i>	involvement of ERCC1/XPF NER endonuclease in regulation of telomere integrity
<i>ERCC4</i>	involvement of ERCC1/XPF NER endonuclease in regulation of telomere integrity
<i>EST1A</i>	even shorter telomeres 1
<i>FBXO4</i>	Fbx4, regulator of Pin2/TRF1 protein abundance, alterations in

	stability can have a dramatic impact on telomere length
<i>FGF2</i>	upregulates telomerase activity
<i>hCLK2</i>	overexpression gradually increases telomere length
<i>HNRPA1</i>	may contribute to maintenance of telomere repeats in cancer cells
<i>HNRPA2B1</i>	telomere maintenance
<i>HSPCB</i>	HSP90 is a key for telomerase activation
<i>hTERT</i>	Catalytic component of telomerase
<i>hTR</i>	Telomerase RNA subunit
<i>IGF1</i>	stimulates telomerase activity
<i>IL15</i>	activates telomerase
<i>IRF1</i>	downregulates telomerase activity
<i>KIP</i>	binds telomerase by interacting with human telomerase
<i>LPA</i>	VEGF dependant induction of telomerase activity
<i>LSMD1</i>	telomere replication
<i>MAPK8</i>	Calcium signalling in ovarian surface epithelial cells induces telomerase activity via JNK
<i>MCRS2</i>	associates with PinX1 to reduce telomere length
<i>NBN</i>	rescue of a telomere length defect of nijmegen breakage syndrome cells requires NBS and telomerase catalytic subunit
<i>NCL</i>	interaction of hTERT and nucleolin participates in the dynamic intercellular localisation of telomerase complex
<i>NCL</i>	nuclear localisation of TERT
<i>NFKappaB</i>	regulates <i>hTERT</i> expression via myc
<i>NMI</i>	inhibits myc induced TERT promoter activity
<i>p23</i>	component of telomerase holoenzyme
<i>PIF1</i>	telomerase regulation
<i>PINX1</i>	binds telomerase so RNA template cannot bind
<i>PKC theta</i>	regulates <i>hTERT</i> expression via myc
<i>POT1</i>	Protection of telomeres 1
<i>PRKCA</i>	Protein kinase C alpha mediates phosphorylation of TEP1 and hTERT to increase telomerase activity
<i>PTGES3</i>	telomerase subunit
<i>RAB9A</i>	upregulated by telomerase
<i>RAD50</i>	telomere maintenance
<i>RAD51L3</i>	telomere maintenance requires RAD51D
<i>RAD54</i>	ALT pathway
<i>RAP1</i>	Telomere binding
<i>RELA</i>	NF-kappa-B p65 modulates telomerase nuclear translocation
<i>RFC1</i>	telomere stability
<i>RIF1</i>	telomere maintenance
<i>RTE1</i>	repressor of telomerase expression 1
<i>RTEL1</i>	regulator of telomere elongation helicase 1
<i>SCYL1</i>	telomerase regulation-associated protein; telomerase transcriptional elements interacting factor
<i>SMC5</i>	Maintains telomere length in ALT cancer cells
<i>SMC6</i>	Maintains telomere length in ALT cancer cells
<i>SMN1</i>	novel telomerase-associated protein, likely to function in human telomerase biogenesis
<i>SMYD3</i>	mediates transcription factor binding to the <i>hTERT</i> promoter

<i>SNRPN</i>	telomere replication
<i>SP1</i>	Sp1 and Sp3 have roles in regulation of human telomerase reverse transcriptase promoter activity
<i>SP3</i>	Sp1 and Sp3 have roles in regulation of human telomerase reverse transcriptase promoter activity
<i>TAK1</i>	represses transcription of telomerase
<i>TELM</i>	telomere length, mean leukocyte
<i>TEP1</i>	telomerase associated protein
<i>TERF2IP</i>	telomeric repeat binding factor 2 interacting protein
<i>TINF2</i>	TRF1 interacting nuclear factor 2
<i>TNKS</i>	tankyrase- releases TRF1 from telomeres and allows access to telomerase
<i>TNKS1BP1</i>	tankyrase 1 binding protein 1
<i>TNKS2</i>	tankyrase 2
<i>TP53</i>	regulation of telomerase activity in ovarian tumours
<i>TRF1</i>	inhibits access of telomerase to telomeres
<i>TRF2</i>	telomere end protection, negative regulator of telomere length
<i>TSN</i>	binds telomere repeats
<i>UBE3A</i>	activates <i>hTERT</i> transcription
<i>VEGF</i>	upregulates telomerase activity
<i>WRN</i>	binding by and stimulation of by telomere binding protein TRF2
<i>WT1</i>	represses transcription of telomerase
<i>XRCC5</i>	Ku70/80 associates with hTERT to regulate access to telomeres
<i>XRCC6</i>	Ku70/80 associates with hTERT to regulate access to telomeres
<i>ZNF146</i>	OZF function may be linked to telomere regulation

### 6.3.2 Microarray analysis of MDS with *del(5q)* with a telomere specific gene set

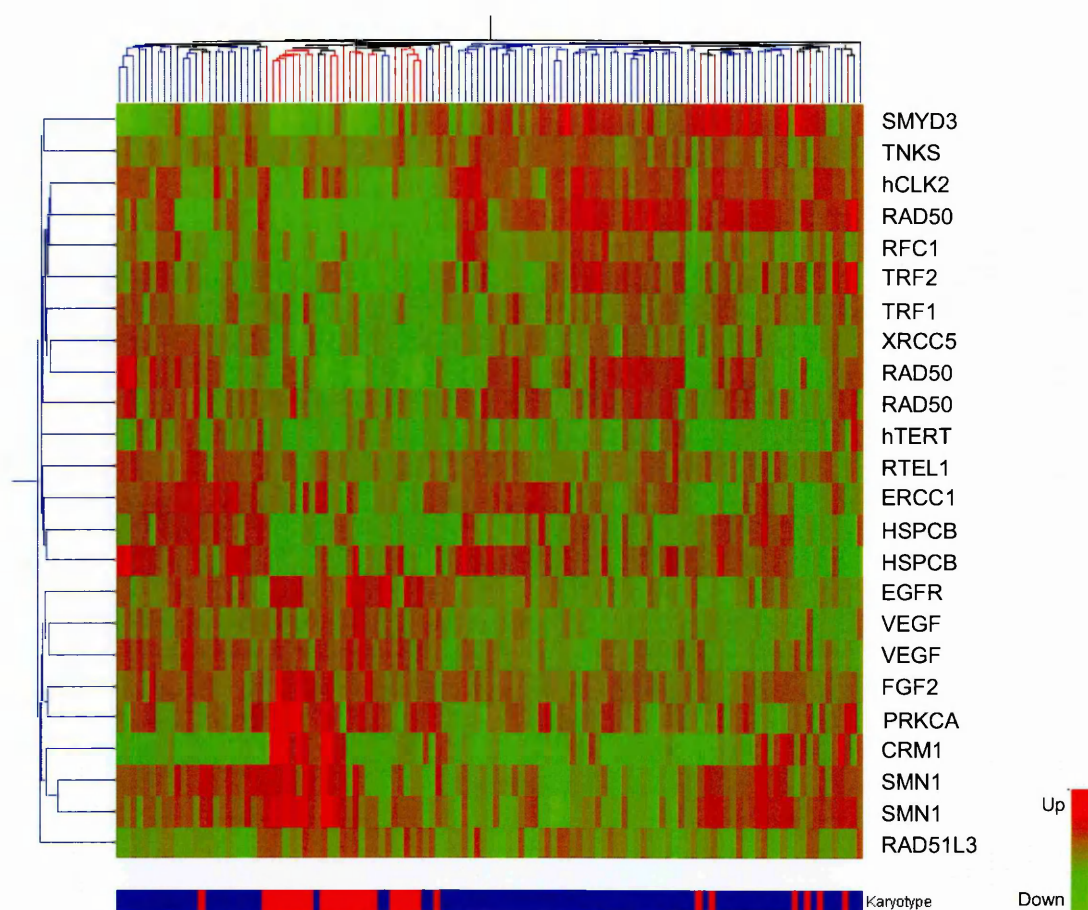
The telomere specific gene set was used for gene expression profiling of CD34<sup>+</sup> cells from patients with MDS. The gene set was used to analyse differences between the CD34<sup>+</sup> cells of patients with and without *del(5q)*. Thirty patients with *del(5q)* and 87 patients with normal or other karyotype were analysed. From the original gene list a set of 19 genes was identified that could distinguish between these two groups. These genes are shown in Table 6.4. Hierarchical clustering was performed with this gene set and it can be seen that some segregation of karyotypic groups occurs (fig 6.2). It can be seen that the majority of patients with *del(5q)* cluster together. However, 23 patients cluster closely and 8 are distributed throughout on separate branches of the tree.

Additionally, the gene set was not able to distinguish normal karyotype MDS from any other karyotype apart from del(5q).

Genes identified by this analysis include several positive regulators of telomere length such as *SMYD3*. This gene was lower in the del(5q) group (range 0.133 – 2.93 median 0.89) than the normal karyotype group range (0.67 – 3.40 median 1.26). Also lower in the patients with del(5q) were *TNKS*, *hCLK2* and *RAD50*, all of which are positive regulators of telomere length. The genes with higher expression in del(5q) tended to be those associated with increased telomerase activity such as *PRKCA*, *FGF2* and *SMN1*.

**Table 6.4 Genes differentially expressed in MDS with and without del(5q)**

Gene Name	Function
<i>CRM1</i>	enhances nuclear export of TERT
<i>EGFR</i>	Antisense epidermal growth factor receptor RNA transfection in human glioblastoma cells down-regulates telomerase activity and telomere length
<i>ERCC1</i>	involvement of ERCC1/XPF NER endonuclease in regulation of telomere integrity
<i>FGF2</i>	upregulates telomerase activity
<i>hCLK2</i>	overexpression gradually increases telomere length
<i>HSPCB</i>	HSP90 is a key for telomerase activation
<i>hTERT</i>	Catalytic component of telomerase
<i>PRKCA</i>	Protein kinase C alpha mediates phosphorylation of TEP1 and hTERT to increase telomerase activity
<i>RAD50</i>	telomere maintenance
<i>RAD51L3</i>	telomere maintenance requires RAD51D
<i>RFC1</i>	telomere stability
<i>RTEL1</i>	regulator of telomere elongation helicase 1
<i>SMN1</i>	novel telomerase-associated protein, likely to function in human telomerase biogenesis
<i>SMYD3</i>	mediates transcription factor binding to the <i>hTERT</i> promoter
<i>TNKS</i>	tankyrase- releases TRF1 from telomeres and allows access to telomerase
<i>TRF1</i>	inhibits access of telomerase to telomeres
<i>TRF2</i>	telomere end protection, negative regulator of telomere length
<i>VEGF</i>	upregulates telomerase activity
<i>XRCC5</i>	Ku70/80 associates with hTERT to regulate access to telomeres



**Figure 6.2 Hierarchical clustering of 24 telomerase related genes in MDS patients with and without del(5q).** Each row represents a single gene and each column a separate patient. The red bars represent patients with del(5q) and the blue bars represent patients without del(5q). The majority of patients with del(5q) cluster together with 8 distributed on separate branches of the tree.

### 6.3.3 Identification of differentially expressed telomere genes in MDS

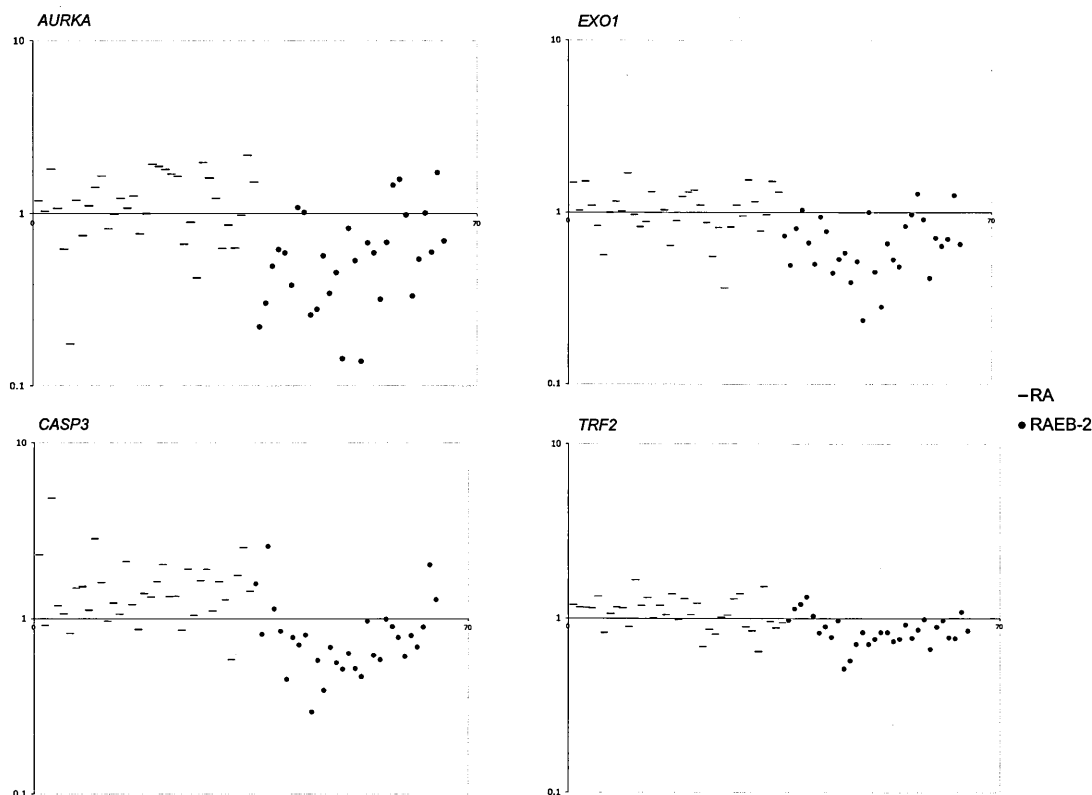
The telomerase specific gene set was used to analyse gene expression differences in patients with distinct disease status. This analysis revealed that the gene set did not give rise to a group of genes that could differentiate between RA, RARS, RAEB-1 or RAEB-2. The two most extreme disease states were compared alone, 35 patients with RA and 30 patients with RAEB-2, and this also did not give rise to a gene set that could

differentiate the two groups. Interestingly, *hTERT* did not differentiate between the RA and RAEB-2 groups. To identify telomerase related genes that may be involved in the pathogenesis of MDS ANOVA was used to compile a list of genes from all probe sets on the array that could distinguish between RA and RAEB-2 using a p value cut off of less than 0.01. This list generated a set of 224 genes that were differentially expressed in RA and RAEB-2. From this list genes were identified that have known function related to telomerase, and genes that are associated with deregulated telomerase or telomere length. Four genes were identified from this list. These are show in Table 6.5 and expression is detailed in Figure 6.3.

**Table 6.5 Telomerase related genes differentially expressed between MDS RA and RAEB-2.**

Gene Name	Function
<i>AURKA</i>	<i>c-Myc</i> upregulation
<i>EXO1</i>	Yeast homolog involved in telomere maintenance
<i>CASP3</i>	Apoptosis. Correlates with <i>hTERT</i> expression
<i>TRF2</i>	Telomere end protection



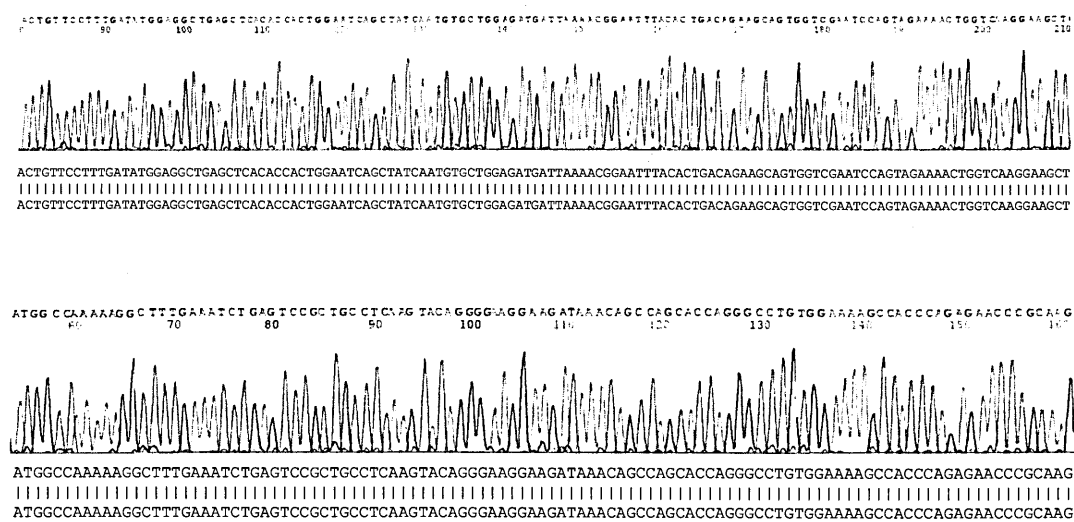


**Figure 6.3** Telomere related genes differentially expressed between MDS RA and RAEB-2. Each dash or triangle represents one patient sample. The line at 1 represents unchanged expression.

#### 6.3.4 *TRF2* gene sequencing

The negative regulator of telomere length *TRF2* was to be sequenced in patients with MDS. Expression of this gene was shown to significantly differentiate between RA and RAEB-2 with RAEB-2 patients having lower expression of the gene. Although reduced expression of a gene is not always associated with mutation there is known to be variation of *TRF2* in exons 3 and 6 (Savage *et al.* 2006). Sequencing of these exons was carried out to identify variation between MDS patients of different disease types and normal controls. Patients with RA, RARS and RAEB and normal controls were sequenced. Genomic DNA was PCR amplified then subjected to cycle sequencing.

Sequence analysis was carried out using the Sequence Analysis software (Informagen). Sequencing data from patients and controls was compared with the known sequence of *TRF2*. In the patient and control samples analysed mutation of *TRF2* was not found. All sequences generated were homologous to the known *TRF2* sequence. An example of the sequence data and alignment is shown in Fig. 6.4.

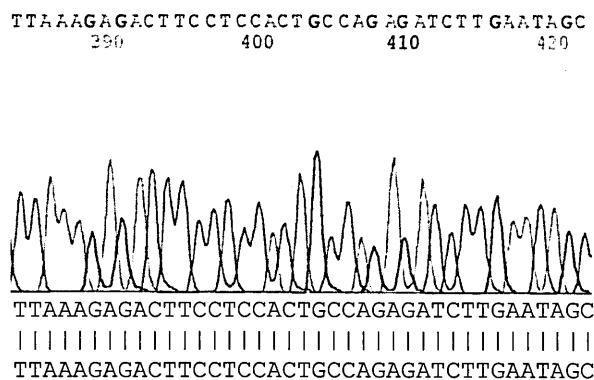


**Figure 6.4** Sequence analysis of *TRF2* in MDS patients. The top panel shows the sequencing electropherogram from one patient sample of *TRF2* exon 3. The alignment of the sequence is shown against the NCBI database sequence. The bottom panel shows the sequencing electropherogram from one patient sample of *TRF2* exon 6. The alignment of the sequence is shown against the NCBI database sequence.

### 6.3.5 *NPM1* gene sequencing

*NPM1* is known to be mutated in exon 12 in 35% of AML patients and of these the majority are normal karyotype. Eighty MDS patients were selected for genomic DNA sequencing of *NPM1* in order to investigate if mutation was present and if it could predict transformation to AML. Initially 19 patients with normal karyotype and RAEB-1 or RAEB-2 were selected then patients were added from other disease groups including 7 with AML arising from MDS. Genomic DNA was PCR amplified then

subjected to cycle sequencing. Sequence analysis was carried out using the Sequence Analysis software (Informagen). Sequencing data from patients and controls was compared with the known sequence of *NPM1*. Sequence analysis revealed that cases of MDS included in the present study did not have mutations in exon 12 of *NPM1* and all sequences corresponded to the database sequence. An example of the sequence data and alignment is shown in Fig 6.5.



**Figure 6.5** Sequence analysis of *NPM1* in MDS patients. Sequencing electropherogram from one patient sample of *NPM1* exon 12. The alignment of the sequence is shown against the NCBI database sequence.

### 6.4 Discussion

Gene expression profiling of CD34<sup>+</sup> cells from MDS patients using the Affymetrix GeneChip U133 plus 2.0 microarray was carried out using a gene set specific for telomere related genes. This list was unable to distinguish between subtypes of MDS. Additionally, according to the array data, the difference in *hTERT* expression between subtypes was not significant.

However, when MDS patients were separated into those with and without a del(5q) 19 genes were differentially expressed between these groups. Although the majority of these patients clustered together 8 were on separate branches of the tree. Genes identified as lower in patients with del(5q) include several positive regulators of telomere length such as *SMYD3*, *TNKS*, *hCLK2* and *RAD50*. *SMYD3* directly activates *hTERT* by binding the promoter and a reduction in *SMYD3* leads to decreased expression of *hTERT* and telomerase activity (Liu *et al.* 2007). Reduction of *SMYD3* has been shown to inhibit cancer cell growth (Wang *et al.* 2008). *TNKS* (Tankyrase) and *hCLK2* positively regulate telomere length and overexpression of *hCLK2* increases telomere length (Smith and de Lange 2000; Jiang *et al.* 2003). *RAD50* is part of the MRN (Mre11–Rad50–Nbs1) complex that is essential for telomere length maintenance and has been reported to be reduced in B-CLL (Poncet *et al.* 2008).

The genes with higher expression in del(5q) tended to be those associated with increased telomerase activity such as *PRKCA*, *FGF2* and *SMN1*. *PRKCA* phosphorylates *hTERT* to increase enzyme activity (Liu 1999). *FGF2* can increase telomerase activity and has been reported to be increased in AML (Kurz *et al.* 2003; Ribatti *et al.* 2007). *SMN1* is a telomerase associated protein that is likely to function to activate telomerase (Bachand *et al.* 2002).

Short telomere length is predictive of poor prognosis in MDS whereas patients with del(5q) have good prognosis. Genes that are associated with an increase in telomere length are decreased in patients with del(5q) suggesting that telomeres would be reduced in these patients. In fact patients with del(5q) and other karyotypic abnormalities have been observed to have shorter telomeres than those patients with normal karyotype (Ohyashiki *et al.* 1994; Sieglöva *et al.* 2004). This could be partly

due to the decreased expression of the telomere length regulators described here. Additionally, decreased expression of, *SMYD3* in patients with del(5q) could be inhibiting cell growth and partly contributing to the good prognosis of these patients. Genes that are associated with an increase in telomerase activity are increased in patients with del(5q) suggesting that they would have an increase in telomerase activity when compared to other MDS patients. At present the amount of data on telomerase activity in patients with del(5q) is limited. Briatore *et al.* analysed telomerase activity in two patients with del(5q) but did not comment on any difference in telomerase activity between these and other MDS patients (Briatore *et al.* 2009). The data presented here suggests that patients with del(5q) have a differential telomerase gene expression pattern when compared to other patients with MDS. Further investigation into the changes in expression of genes that control telomere length and telomerase activity and analysis of telomerase activity and *hTERT* expression itself in patients with del(5q) will be of interest to further understanding of the importance of telomerase regulation in these patients.

Investigation of the most statistically significant differentially expressed genes between subtypes of MDS indicated some involvement of telomerase related genes, namely *AURKA*, *EXO1*, *CASP3* and *TRF2*.

*AURKA* (aurora kinase A) shows decreased expression in patients with RAEB-2 MDS when compared to those with RA. *AURKA* has been reported as overexpressed in many cancers including breast, colorectal, gastric and pancreatic (Lens *et al.* 2010). The gene can be amplified in cancers and overexpression is associated with poor prognosis and genomic instability (Lens *et al.* 2010). In breast cancer it is frequently overexpressed and is known to induce telomerase activity through upregulation of *c-MYC* (Yang *et al.* 2004). However, *c-MYC* expression appears to be increased in the

CD34<sup>+</sup> cells of patients with MDS (Sloand *et al.* 2007; Vasikova *et al.* 2010). *c-MYC* is regulated by many proteins and in MDS cases *AURKA* may not be effecting *c-MYC* expression to regulate *hTERT*. In AML expression of *AURKA* is varied but those patients that have high expression have unfavourable cytogenetics (Lucena-Araujo *et al.* 2011). Mutation of the gene can lead to decreased expression and this is thought to increase risk of oesophageal cancer (Lens *et al.* 2010). This suggests that decreased expression of *AURKA* can also lead to cancer and, although the gene may not be affecting *hTERT*, this may be the case in MDS.

*EXO1* has been studied in yeast and has been shown to directly contribute to telomere shortening (Bertuch and Lundblad 2004). Interestingly, in the absence of telomerase *EXO1* was shown to enhance telomere function and cell proliferation by a telomerase independent pathway. No data is available on the function of human *EXO1* on telomeres at present. If the function of human *EXO1* is related to that of yeast then it could be that the decrease in *EXO1* associated with MDS RAEB-2 allows some telomere maintenance to occur, keeping telomere length above the critical level and allowing cells to extend their proliferative potential.

Oshima *et al.* demonstrated that *CASP3* (pro-apoptotic) is expressed in MDS in higher levels than in normal control cells (Ohshima *et al.* 2003). This was detected by immunohistochemistry of bone marrow biopsies. However, they did not differentiate between disease subtypes. They show that *CASP3* expression coupled with a decrease in *hTERT* expression is associated with poor prognosis. However, in the study no interaction between these genes was identified. This correlation may be by chance. In a later study *CASP3* was identified as significantly decreased in RAEB-2 patients when compared with RA patients (Pellagatti *et al.* 2006). *CASP3* is a downstream target of

TRF1 and overexpression of TRF1 increases CASP3 (Kishi *et al.* 2001). Reduced TRF1 could be partly causing the reduced CASP3 and allowing cells to continue to proliferate. In the current study CASP3 is reduced in the patients with RAEB2 suggesting that there would be a reduction in apoptosis in these patients as has been documented (Parker *et al.* 1998).

The absence of mutation of TRF2 in patients with MDS shows that mutation is unlikely to be responsible for the reduced expression of TRF2 in patients with RAEB2. However, this reduced expression is still of interest. Decrease in expression of TRF2 may lead to reduced binding of TRF2 at the telomere allowing telomerase to access the telomere repeats and lengthen shorter telomeres (Smogorzewska *et al.* 2000). TRF2 is known to function in telomere end protection so a dramatic reduction of TRF2 could leave telomere ends unprotected and activate the DNA damage response (de Lange 2002).

Although the number of telomerase related genes differentially expressed between RAEB-2 MDS is small they indicate that regulation of telomerase and telomeres may contribute to disease progression. Reduction of TRF2 and EXO1 contribute to telomere maintenance allowing telomere length to be maintained above the critical level. This is complemented by the reduction of CASP3 that leads to a reduction of apoptosis in these cells.

NPM1 exon 12 mutations are common in patients with AML. Patients with MDS are known to progress to AML. Eighty patients with MDS were tested for NPM1 exon 12 mutations to see if the presence of the mutation could predict disease progression or prognosis in MDS. However, NPM1 mutations were not demonstrated in patients with

MDS. This result is in agreement with several subsequent studies that used sequence analysis to assess the mutation status of *NPM1* in MDS. These studies involved smaller sample size than the present study and the majority reported no mutations of *NPM1* in MDS (Caudill *et al.* 2006; Oki *et al.* 2006; Shiseki *et al.* 2007; Zhang *et al.* 2007; Dicker *et al.* 2010). The study by Zhang *et al.* showed two MDS patients out of 38 analysed had mutated *NPM1* (Zhang *et al.* 2007) and they suggest that the mutation may be involved in the pathogenesis of MDS. Dicker *et al.* report one of 66 MDS patients in their study had mutated *NPM1* (Dicker *et al.* 2010). This group also reported a low incidence (8.5%) of *NPM1* mutation in AML arising from MDS and suggest a different disease mechanism for de novo AML in which 25% of cases are mutated. Two recent studies analysed larger cohorts of patients and report a higher incidence of mutation (Li *et al.* 2010; Bains *et al.* 2011). Li *et al.* report that nine of 232 patients have a mutation while Bains *et al.* found seven of 160 patients with mutated *NPM1*. In the study by Bains *et al.* no cases with only mutated *NPM1* progressed to AML and authors suggested that the mutation is not sufficient to explain progression of MDS to AML. However, they did report that when *NPM1* is mutated in combination with *FLT3* mutation all cases (four) progressed to AML and suggest these mutations are useful markers for predicting progression of MDS to AML. Taken together with the present investigation all of the above studies total an analysis of 684 MDS patients. This indicates that 2.7% of MDS patients could carry the mutation. The low incidence of mutation of *MPN1* in the combined studies indicates that mutation of this gene as a sole genetic abnormality is unlikely to be an important prognostic indicator in the progression of MDS to AML. However, a recent study has reported *NPM1* expression is reduced in patients with advanced del(5q) MDS and authors suggest that this decrease in expression contributes to the pathogenesis of advanced stage MDS by contributing to genomic instability (Pellagatti *et al.* 2011).



Disease progression in MDS is clearly complex but the data presented here suggest that differential expression of genes associated with telomerase and telomere maintenance may be associated with the pathogenesis of MDS, in particular those patients with a del(5q).

# ***Chapter 7***

## ***General Discussion***

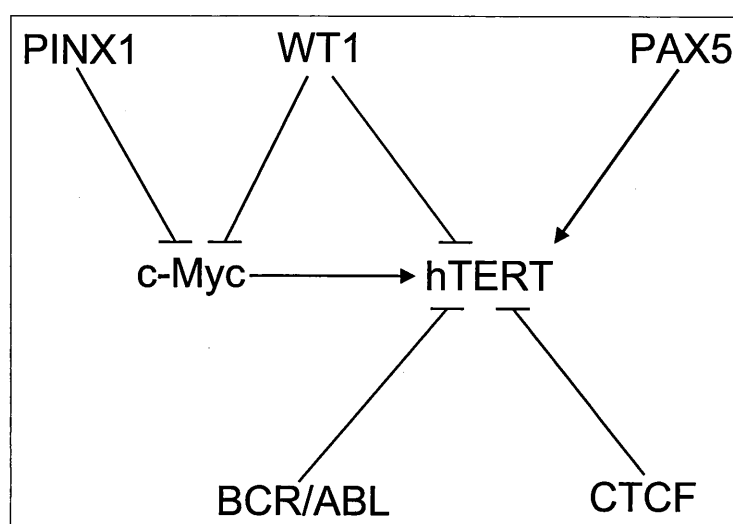
Real time PCR has been used to investigate gene expression levels of individual telomerase associated genes in CML. This analysis revealed that, in contrast to many studies of human cancer, *hTERT*, the catalytic component of telomerase is down regulated in patients with CML. Furthermore, expression is reduced with disease progression. This finding has many implications in the field of telomere biology, particularly with the advent of telomerase inhibitors for treatment of cancer.

Expression of *hTERT* was measured in combination with other telomerase related genes *hTR*, and *TEP1*, (subunits of telomerase), *TRF1*, *TRF2* and *Tankyrase*, (telomeric-associated proteins) and *c-Myc* and *PinX1*, (positive and negative regulators of telomerase, respectively).

The reduced expression of *hTERT* in CML is likely to be caused by many interacting factors, several of which have been analysed in the present study. Interaction of these factors with *hTERT* is summarised in Fig 7.1. It is likely that the reduced expression of *hTERT* observed in the current study is partly due to the downregulation of *c-Myc*, which positively controls expression of *hTERT*. Interestingly, it has been shown by immunohistochemical methods that *c-Myc* expression in CML was lower in BC than in AP, this is in agreement with the current study (Vidovic *et al.* 2008).

Expression of *c-Myc* may be influenced by the expression of *PinX1*. Transfection of *PinX1* into *PinX1* negative gastric cancer cells reduced expression of *c-Myc* and telomerase activity (Wang *et al.* 2010). Furthermore, RNAi knockdown *PinX1* resulted in increased expression of *c-Myc*. Therefore, the presence of elevated *PinX1* expression above normal control levels could contribute to the resulting decrease in *c-Myc* expression which in turn leads to decreasing *hTERT* expression. It may also be

suggested that this decrease in telomerase is a result of the *BCR/ABL* fusion gene in CML. In fact, it has been shown that antisense inhibition of *BAR/ABL/c-ABL* increases *hTERT* levels (Bakalova *et al.* 2004). Therefore, the presence of the *BCR/ABL* fusion in CML may contribute to the down regulation of *hTERT* observed in the CD34<sup>+</sup>/blast cells of the CML patients in this study. If this is the case it may be that the downregulation of *hTERT* in CML may be more pronounced in this disease as a result of the *BCR/ABL* fusion.



**Figure 7.1 Regulation of *hTERT* expression in CML.** WT1 and PINX1 negatively regulate *c-Myc* expression which results in decreased expression of *hTERT*. WT1 and *BCR/ABL* also decrease expression of *hTERT*. Reduction in *PAX5* expression could decrease telomerase expression. In the absence of promoter methylation CTCF inhibits *hTERT* expression.

It appears that there are still many regulators of *hTERT* to be assessed and new candidate genes are being identified regularly. Recently *PAX5*, a B-cell specific transcription factor has been identified as an activator of transcription of *hTERT* (Bougel *et al.* 2010). The binding site of *PAX5* in the telomerase promoter was identified by EMSA and ChIP experiments. It was demonstrated that ectopic expression of *PAX5* in a telomerase negative cell line was sufficient to activate *hTERT*

transcription. As *PAX5* expression is normally restricted to B cells it would be of interest to investigate expression of *PAX 5* in CML. *PAX5* is expressed on pre-B cells which are CD34<sup>+</sup>.

The Wilms' tumour 1 (*WT1*) gene was initially identified as a tumour suppressor gene but it has a number of target genes and has also been shown to have oncogenic effects. *WT1* binds both the *hTERT* and *c-Myc* promoters and expression of *WT1* results in a reduction of *hTERT* expression (Sitaram *et al.* 2010). *WT1* is known to be overexpressed in CML and has shown to be increased with disease progression (Inoue *et al.* 1994; Gerber *et al.* 2011). Further studies of *WT1* in CD34<sup>+</sup> cells in combination with measurement of expression levels of *hTERT* and other telomerase associated genes could provide further insight into the regulation of telomerase in CML.

It was revealed that in CML the *hTERT* core promoter was not methylated and the absence of methylation could lead to the repression of *hTERT* transcription by the binding of CTCF which would lead to the observed reduction in transcription. Further investigation of this interaction is warranted and the entire CTCF binding site in the promoter could be investigated by bisulfite sequencing. This will allow analysis of individual CpG sites of the *hTERT* promoter and full characterization of the methylation status of the CTCF binding site. Recently DNA methylation microarrays have been used in combination with a methylated CpG island recovery assay to analyse genome wide methylation patterns in ALL (Dunwell *et al.* 2010). In the same study this group then analysed the genes that were frequently methylated in ALL in patients with CML to see if methylation correlated. They showed that methylation of this set of genes was increased in CML with disease progression. This study only analysed a small set of genes in CML patients and this showed a correlation with disease progression suggesting that methylation does play a role in progression of CML. Further studies of

CML patient DNA methylation using whole genome CpG arrays could provide further clues into the specific genes involved in disease progression.

Patients with ET exhibiting the *JAK2* V617F mutation were studied by cDNA microarray. It was shown the neutrophils from these patients were not heterogeneous when compared to normal neutrophils suggesting that the presence of the mutation does not have a major effect on gene expression changes in neutrophils and that the granulocyte lineage may not be involved in the pathogenesis of ET. It has been shown that *JAK2* V617F positive myeloproliferative neoplasms can often give rise to AML that is negative for the mutation further suggesting that the presence of the *JAK2* V617F alone is not sufficient for transformation (Theocharides *et al.* 2007). Genetic abnormalities in the neutrophils of patients with ET have been investigated by SNP microarray analysis in a study by Kawamata *et al.* (Kawamata *et al.* 2008). This study demonstrated that genomic abnormalities were rare in ET but the abnormalities observed were only in those patients with mutated *JAK2*. Three abnormalities were identified in three separate patients: Deletion of 5q23.1, Trisomy 9 and uniparental disomy at 9p. Uniparental disomy of 9p is known to be a mechanism of duplication of the mutated *JAK2* allele (Kralovics *et al.* 2005) and trisomy of chromosome 9 could also be viewed to have this function. Further investigation of genetic changes in patients with ET in combination with gene expression profiling could provide further clues into the pathogenesis of this disease.

Expression of telomere related genes was also investigated in MDS by microarray analysis. A subset of genes were identified that could differentiate between those patients with and without a del(5q). Genes identified as lower in patients with del(5q) include several positive regulators of telomere length such as *SMYD3*, *TNKS*, *hCLK2*

and *RAD50*. The genes with higher expression in del(5q) tended to be those associated with increased telomerase activity such as *PRKCA*, *FGF2* and *SMN1*. This suggests that in these patients telomere length would be decreased but this is counteracted by increased expression of genes promoting telomerase activity. Direct measurement of *hTERT* expression in these patients would provide insight into the role of this gene in the prognosis of these patients.

Genes that could differentiate between RA and RAEB2 subtypes were mostly related to telomere maintenance. Of those genes identified *TRF2* and *EXO1* are known to have functions that negatively regulate telomere length while *AURKA* can increase *hTERT* expression and *CASP3* promoted apoptosis. Reduction of *TRF2* and *EXO1* contribute to telomere maintenance allowing telomere length to be maintained above the critical level. This is complemented by the reduction of *CASP3* that leads to a reduction of apoptosis in these cells. The patients with reduced expression of these genes were patients with RAEB2 which is also associated with leukaemic transformation suggesting that deregulation of this set of genes may be associated with disease progression.

Genes involved in expression and regulation of *hTERT* do not appear to play a major role in disease progression of MDS, in contrast to disease progression of CML. Rather, it is telomere length that seems to be important. Reduced telomere length has been shown to be important for disease progression of MDS. Erosion of telomeres is known to contribute to genetic instability by increasing the number of chromosome end to end fusions, especially when *TRF2* is reduced. The reduction in *TRF2* expression in RAEB2 MDS suggests that the telomere capping function of *TRF2* will be reduced allowing an increase in chromosome end to end fusions and non reciprocal

translocations which are characteristic of genomic instability. The resulting instability then leads to tumour formation. It is likely that for MDS cells to continue dividing they have lost cell cycle checkpoint control. Indeed, it has been reported that mutations in *TP53* are present in up to 25% of primary MDS patients and preferentially in those patients with RAEB (Ludwig *et al.* 1992; Sugimoto *et al.* 1993; Wattel *et al.* 1994; Kaneko *et al.* 1995). p53 would normally cause a cell to enter cell cycle arrest after DNA damage such as chromosome fusions. Mutation of the gene inactivates the protein and allows the cell to continue cycling permitting further telomere erosion and genomic instability. Therefore it could be suggested that mutation of *TP53* or a defect in another cell cycle checkpoint could allow the cells of MDS patients to continue cycling and result in decreased telomere length, which in conjunction with reduced *TRF2* expression leads to chromosome end to end fusions. No data on *TP53* gene status was available for the patients included in the present study but correlation of this with expression of *TRF2* could prove useful to predict those patients at greater risk of genomic instability and disease transformation. It is thought that telomerase is required to confer immortal growth on to cells that exhibit instability and it has been shown that *hTERT* is expressed in MDS.

Perhaps the mechanisms of disease progression in CML and MDS are similar; both have reduced telomere length which will increase genetic instability and lead to disease progression. However, in CML the presence of the BCR/ABL fusion product and deregulation of several telomerase associated genes appears to contribute to reducing *hTERT* expression. This is unlikely to be the case in MDS. To further elucidate the importance of telomerase in MDS direct measurement of *hTERT* and telomerase associated genes in the CD34<sup>+</sup> cells of patients with specific disease subtypes and karyotypic abnormalities would be valuable.



The data in the current study challenge the view that telomerase is always increased in cancer. It is accepted that most tumours express *hTERT* and are telomerase positive, but it is not usually clear whether the observable *hTERT* expression is due to their origin from telomerase positive stem cells or due to reactivation of the gene during tumorigenesis. Such studies, which have compared *hTERT* or telomerase activity in tumour cells with corresponding normal tissues, and not the normal stem cells of origin, have given rise to the observation that tumour cells have increased levels of telomerase. However, in solid tumours it is not possible to compare tumour cells with their corresponding stem cells as it is for leukaemia cells with CD34<sup>+</sup> stem cells. The data presented here demonstrates that the CD34<sup>+</sup>/blast cells of patients with CML possess lower *hTERT* expression levels than CD34<sup>+</sup>/blast cells from normal individuals. Like other stem cells the telomeres of normal CD34<sup>+</sup> cells are maintained by low levels of telomerase and show only gradual telomere shortening with ageing and rapid cycling. Telomerase activity ensures that the stem cell compartment will be able to respond adequately to large expansion demands and, whilst the telomeres still gradually shorten, the time to critically shortened length is delayed by telomerase. In CML the turnover of all types of Philadelphia positive stem and progenitor cells is known to be increased and several studies suggest that telomere length measurements may be a useful indication of stem cell turnover *in vitro* and *in vivo* (Brummendorf *et al.* 2000). This result suggests that telomerase may not always be increased in malignancy and that the reduction of *hTERT* expression in CML directly causes telomeres to shorten as the cells divide in order to increase genetic instability and drive disease progression. Whether this phenomenon is specific to CML cannot yet be speculated. However, recently it has emerged that other malignancies do not have increased *hTERT* expression or telomerase activity. This is particularly of note in Chronic Lymphocytic Leukaemia (CLL). It has been demonstrated by expression analysis that *hTERT* expression is more than two fold

reduced in CLL patients when compared with normal controls (Poncet *et al.* 2008). More recently, reduced telomerase activity and telomere dysfunction have been identified in CLL (Lin *et al.* 2010). In both of these studies B-cell populations were isolated from patients and normal controls, allowing a direct comparison of the malignant cells with the cells of origin. This further demonstrates the importance of comparing like with like when analysing malignant cells.

Although more difficult to isolate in solid tumours Liu *et al.* have analysed homogenous populations of cells in breast carcinomas (Liu *et al.* 2004). Using mRNA in situ hybridisation they were able to compare expression of *hTERT* in only the cells of interest. This showed that expression of *hTERT* was high in normal breast tissue but significantly reduced in ductal carcinoma in situ.

Reduction of telomerase activity has also been demonstrated in B-cell lymphomas. Klapper *et al.* showed that telomerase activity was not significantly different from that of normal controls in mantle cell lymphoma, follicular lymphoma and diffuse large B cell lymphoma (Klapper *et al.* 2003). They were able to demonstrate significantly increased telomerase activity in Burkitt's lymphoma which is characterised by the t(8;14) translocation which results in overexpression of *c-Myc*. This increase in expression of *c-Myc* is thought to directly cause the increase of telomerase activity in Burkitt's lymphoma. This suggests that lymphomas do not show increased telomerase activity unless *c-Myc* is overexpressed.

The suggestion that telomerase is not increased in all malignancies has implications for the study of telomerase inhibitors as treatment for cancers. These drugs are designed with the idea that telomerase is increased in cancer cells and inhibition of telomerase is

expected to reduce telomere length and lead to death of the cancer cells. Telomerase inhibitors could have adverse effects on those cells that express telomerase normally, such as haematopoietic stem cells. It could be suggested that telomerase inhibitors may deplete, for example in the bone marrow, haematopoietic stem cells and not the low telomerase expressing cancerous cells thereby giving the cancer cells a growth advantage over the normal cells. It has even been suggested that telomerase inhibition could lead to an increase in genomic instability in surviving malignant cells and result in a more aggressive disease phenotype. Phase 1 clinical trials of oligonucleotides targeting hTR binding are currently being carried out in patients with CLL and other lymphoproliferative diseases (ClinicalTrials.gov identifier NCT00124189). As there is evidence of reduced *hTERT* expression in CLL it will be of great interest to see the results of this study especially in relation to the cell types that have been affected and progression of disease.

Mechanisms of telomerase regulation in malignancy are clearly complex and many factors influence both telomere length and *hTERT* expression. The data presented here show that reduced expression of *hTERT* and other telomerase associated genes is important for disease progression in CML and some other cancers. Whether this is the case with many other malignancies is not yet known but additional studies of cancer stem cells will allow further investigation of this important finding. Further studies comparing gene expression in solid tumours with their cells of origin may increase understanding of this issue.

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